Reducing the Toxicity of Autologous Haemopoetic Stem Cell Transplants

by

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Contents

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Abstract

Post-thaw washing of cryopreserved blood stem cells is intended to reduce side effects and is an increasingly common practice in Autologous Stem Cell Transplantation (ASCT). However, the potential advantages of this extra manipulation have not been fully evaluated, and needs to be balanced against the fact that patient morbidity has already been significantly lowered by reductions in the reinfusion volume (achieved through refined harvesting, mobilising and processing techniques) and improved patient pre-medication. This study modified and validated the New York Cord Blood Banks washing protocol (Rubinstein et al. 1995). Parameters validated included; cell loss, overall viability, viable $CD34^{+}$stem cell enumeration, clonogenic potential and reduction in free haemoglobin content. The results demonstrate that washing of cryopreserved stem cells improves the outcome of several key laboratory parameters without a significant negative impact upon haematopoietic stem cell (HSC) engraftment potential.

To determine whether a clinical need exists for the routine washing of cryopreserved HSC autografts, a single institution, non-randomised clinical trial was performed. Patients who met trial eligibility criteria were consented for the washing of their ASCT graft, whereas all other transplant patients received thawed unwashed blood stem cells in accordance with current practice. Reinfusion associated side effects experienced by patients, their close family members (carers) and nurses following the infusion of washed or unwashed stem cells were assessed using both a Quality of Life (QOL) survey and nursing documentation forms. The surveys were self-completed by the patient, the nurse and the carer at intervals over a 24 hour period. Statistical analysis of the QOL data demonstrated that washing was associated with an improvement in several side effects associated with reinfusion including flushing, nausea and unpleasant tastes for patients and unusual smells for both carers and nurses. Furthermore, the clinical observations indicate that the washing protocol reduces transplant associated hypertension in ASCT patients and is an improvement on the reinfusion of unwashed HSC, which has a significant correlation with increased blood pressure.

Consequently, this study has identified a number of circumstances in which a post-thawing washing of HSC autografts is potentially beneficial for the patients. The washing protocol reduces the amount of cellular debris and DMSO reinfused as well as diminishing patient discomfort and therefore should be considered in the event of large volume reinfusions, patient allergies or where hemodilution may exacerbate existing cardiac issues. Furthermore, post-thaw washing of HSC should also be contemplated in the event of unexpected delays between autograft thawing and reinfusion as a strategy for extending the viability of HSC.

Foreword

Autologous stem cell transplantation (ASCT) after high dose chemotherapy is increasingly being used as a treatment for acute leukaemia, lymphoma and solid tumours and requires the cryopreservation and storage of autologous stem cells harvested from bone marrow or peripheral blood during remission. The stem cells are frozen in liquid nitrogen and subsequently stored at ultra-low temperatures until required for a transplant, sometimes years later. In order to prevent damage to the stem cells by ice crystals a cryoprotectant, dimethyl sulfoxide (DMSO) is added to the cells prior to freezing and is subsequently reinfused into the patients along with the cellular contents. A number of DMSO-related toxicities has been reported including headache, nausea, abdominal cramping, flushing, decreased heart rate, neurological toxicity and increased blood pressure. Similarly, red blood cells are destroyed during the freezing process, and the reinfusion of red cell lysis products exert additional toxic effects to the patient.

Whilst the toxic side effects of DMSO infusion are well known, there has been significant concern that removing this compound prior to transplant will reduce stem cell viability and numbers and hence increase the risk of graft failure.

This study was undertaken to validate a method of removing DMSO and cellular lysis products from thawed autologous stem cell collections without comprising the engraftment potential and to assess the impact on patient well-being using a quality of life survey.

Commonly Used Abbreviations

ABMT	autologous bone marrow transplant
ACDA	acid citrate dextrose anticoagulant
AL	amyloid light-chain
AML	acute myeloid leukaemia
ASCT	autologous stem cell transplantation
ABMTRR	australasian bone marrow transplant recipient registry
BFU-E	burst-forming unit erythroid
BM	bone marrow
BMH	bone marrow harvest
$CD34^+$	cluster of differentiation 34 positive
CFU-GEMM	colony-forming unit granulocyte erythroid monocyte megakaryocyte
CFU-GM	colony-forming unit granulocyte-macrophage
CML	chronic myeloid leukaemia
DMS	dimethyl sulfide
DMSO	dimethyl sulfoxide
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
G-CSF	granulocyte colony-stimulating factor
GVHD	graft versus host disease
ISHAGE	international society of hematotherapy and graft engineering
ISCT	international society of cellular therapy
HD	hodgkin's disease
HLA	human leucocyte antigen
HSC	haematopoietic stem cell
MHC	major histocompatibility complex
MM	multiple myeloma
MDS	myelodysplastic syndromes
NYCBB	new york cord blood bank
NHL	non-hodgkins lymphoma
NCC	nucleated cell concentration
PB	peripheral blood
PBSC	peripheral blood stem cell collection
PE	phycoerythrin
SAA	severe aplastic anaemia
t-AML	therapy-related acute myeloid leukaemia
t-MDS	therapy related myelodysplasia
TBI	total body irradiation
TNC	total nucleated cell count
UCB	umbilical cord blood
WCC	white cell count
7-AAD	7-amino-actinomycin D

Chapter 1

Literature Review

1.1 Haematopoietic Stem Cell Transplantation

Autologous stem cell transplantation (ASCT) is an established clinical practice, frequently used in the treatment of lymphomas, leukaemias and solid tumours. It involves the intravenous reinfusion of the patients haematopoietic stem cells (HSC), obtained from bone marrow (BM) or peripheral blood (PB) following high dose chemotherapy. These stem cells are harvested from the patient preceding the transplant and are stored at 196C in liquid nitrogen until required. Prior to freezing, the stem cells are treated with a chemical called dimethyl sulphoxide (DMSO), which prevents cell death during the freezing and thawing process. At the actual transplant, the patient is reinfused with the entire contents of the thawed harvest; that is, stem cells, DMSO, and blood cell breakdown products. This review considers the toxicity of the reinfused products and its effects on patient well being.

1.1.1 Historical Perspective

The first recorded use of BM to treat disease occurred in 1891 (Brown-Sequard and d'Arsonaval) with the oral administration of glycerol extracts of BM to anaemic patients suffering from leukaemia (reviewed by [Johnson, 1981]). Attempts at finding the best method of administering cells continued into the early 20th century with the use of intramuscular injections of freshly prepared BM (Schretzenmayr 1937) as an unsuccessful treatment of anaemia related to malaria (reviewed by [Santos, 1983]). In 1944, following 5 years of investigation Jean Bernard reported that injections of small volumes of BM ($2.0-6.0$ ml) directly into the medullary cavity were ineffective for cellular transplan-

tation (reviewed by [Johnson, 1981]). The first report of intravenous administration of BM [Osgood et al., 1939] was for the unsuccessful treatment of aplastic anaemia using 13mls of BM collected from a sibling. It wasn't until the mid 1940s however that intravenous BM administration was accepted, as the most efficient and effective means of transplantation [Santos, 1983].

Post world war two investigations into the effect of irradiation resulted in the discovery [Jacobson et al., 1951] that erythrocyte production could be restored in mice, following the destruction of BM by lethal dose radiation, if haematopoietic tissue, such as the spleen were shielded from the radiation. In addition further studies showed that the haematopoietic function was restored in lethally irradiated mice and guinea pigs by intravenous or intra-peritoneal injections of allogeneic or syngeneic BM [Lorenz et al., 1951] contributing to the advancement of this field. Subsequent studies [Lindsley et al., 1955, Nowell et al., 1956] showed that the colonisation of the recipient BM was due to the transplanted cells rather than to a humoral effect.

1.1.2 Types of Transplants

Autologous

Autologous transplantation involves the intravenous infusion of the patients own HSCs, collected from BM or PB, to replace diseased BM or to replenish terminally damaged BM after the use of myeloablative therapy [Armitage, 1994]. The observation that the administration of the patients' own BM (stored prior to irradiation) or BM from an identical twin resulted in the restoration of the patients' marrow, suggested the possibility that autologous BM could be stored in anticipation of radiotherapy, chemotherapy or the possibility of radiation accident [Thomas et al., 1957]. The development of cryopreservation techniques [Mannick et al., 1960, Cavins et al., 1962, Buckner et al., 1970, Malinin et al., 1970] for the storage of HSC at ultra low temperatures ensured the acceptance of autologous BM transplantation (AutoBMT) as a therapeutic practice for the treatment of lymphoma in the 1970s [Armitage, 1994].

From the 1980s onwards, ABMT has been increasingly used in the treatment of a variety of diseases including; acute myeloid leukaemia (AML), lymphoproliferative disorders, multiple myeloma (MM) and solid tumours. These will be discussed in further detail in section 1.2 of this review [Apperley et al., 2000, Thomas, 2000]. Generally the upper age limit for autologous transplantation is 65 years although the patients health and disease status are also important considerations [Apperley et al., 2000].

As Graft Versus Host Disease (GVHD) is not a complication, transplant mortality for autologous transplantations is generally negligible, although this procedure has its own risks, which will be outlined in section 1.3 [Nivison-Smith et al., 2005].

Allogeneic

Allogeneic transplantation involves the transfer of HSCs, collected from the BM or PB, of an HLA-matched related or unrelated donor, into another person [Armitage, 1994]. Umbilical cord blood (UCB) is also a potential source of allogeneic HSC [Kurtzberg et al., 1996].

In comparison to BM or PBSC, USB transplants (UCBT) have several advantages including a lower risk of severe graft versus host disease, rapid availability (from cord banks), lack of risk for donor and lower risk of infectious diseases [Barker et al., 2005] [Brunstein and Wagner, 2006]. However the disadvantages of UCBT include the inability to "recollect" from a donor in the event of disease progression or relapse, lack of substantial experience with UCBT in comparison with the transplantation of HSCs derived from BM or PB and most significantly the limitation of cell dose which has resulted in UCB generally been restricted to the treatment of children [Brunstein and Wagner, 2006]. Nonetheless, in the future the use of UCB may be routinely expanded to adult patients as transplantation of two ("double") partially HLA-matched UCBs may overcome the restrictions of cord blood cell dose [Barker et al., 2005]. However as phase II clinical trials of "double" adult UCB transplants are ongoing, more data needs be collected and analysed before this form of HSC allogeneic transplant may be adopted as standard practice worldwide for adult patients [Brunstein and Wagner, 2006].

Early attempts at allogeneic transplantation were followed by a severe and rapidly fatal reaction characterised by rashes, diarrhoea and pneumonitis, which proved to be a major obstacle in the development of this form of transplantation [Storb et al., 1970, Thomas et al., 1975, Santos, 1983]. GVHD, occurs when allogeneic BM recognises the tissues of the recipient as foreign [Janeway et al., 1999].

The identification of the major histocompatibility complex (MHC) HLA system and the subsequent ability to type and match class I and II antigens for donors and recipients has contributed to a reduction in graft versus host disease [Apperley et al., 2000, Kollman et al., 2001]. In addition, the adoption of immunosuppressive agents such as

- methotrexate [Storb et al., 1970],

- cyclophosphamide [Thomas et al., 1975] and
- cyclosporine A [Ruutu et al., 2000]

has resulted in the improved management of GVHD and improved allogeneic transplant outcome [Sullivan and Storb, 1984].

Allogeneic transplants may be considered for the treatment of a number of diseases including: acute and chronic leukaemias, malignant lymphomas, MM, myelodysplastic syndromes (MDS) and severe aplastic anaemia (SAA) [Sullivan and Storb, 1984, Apperley et al., 2000, Nivison-Smith et al., 2005]. A major consideration in the decision for allogeneic transplantation is the age of the recipient, as there is a increased frequency of GVHD with age [Armitage, 1994]. As a general rule 60 years is the age limit for the use of HLA-identical sibling donors and 45 years the age limit for unrelated donor transplants [Apperley et al., 2000].

The availability of compatible donors as well as the individual patient's health and disease status are also major considerations in the decision to perform allogeneic transplant [McGlave, 1985, Nivison-Smith et al., 2005]. This form of HSC transplantation initially has higher transplant related mortality and initial worse survival and disease free survival in comparison to autologous procedures. On the other hand, unlike autologous transplants allogeneic transplants are potentially curative [Armitage, 1994]. Today the number of autologous transplant procedures performed exceeds allogeneic procedures as increasing applications are found for this procedure [Apperley et al., 2000, Nivison-Smith et al., 2005].

1.1.3 Sources of Autologous Transplant Material

Bone Marrow

As the site of HSC production, BM was the original source of HSCs and it remains an acceptable source today. Harvesting BM to obtain HSCs involves multiple aspirations from the iliac posterior crests, in an operating theatre, under sterile conditions and general anaesthesia [Thomas and Storb, 1970] until sufficient mononuclear cells have been removed, ideally 3×10^8/kg [Apperley et al., 2000]. The development of recombinant cytokines such as granulocyte colony-stimulating factor (G-CSF) [To et al., 1998] resulted in mobilisation of HSCs, increasing BM harvest yields including those of patients who had received previous cytotoxic chemotherapy [Tegg et al., 1999].

Peripheral Blood

The presence of HSC in blood was recognised in animals and humans in the 1960s [Goodman and Hodgson, 1962, McCreddie et al., 1971] however it was not until the 1980s that PB HSC harvests were performed on humans. Initially there were difficulties obtaining sufficient cells for transplantation by aphaeresis, as levels of HSCs in PB were approximately 100 times less than in BM [Gillespie and Hillyer, 1996]. HSC yields were significantly improved, however, by timing PB collections to coincide with white cell count (WCC) increases, post recovery from myelosuppressive chemotherapy [To et al., 1984, Juttner et al., 1985, To et al., 1990, To et al., 1997]. Furthermore, the use of haematopoetic growth factors such as G-CSF and stem cell factor (SCF) to mobilise HSCs [Molineux et al., 1990] also resulted in dramatic increases in HSC numbers [de Revel et al., 1994]. Today PB collections are generally primed with G-CSF and/or chemotherapy to ensure adequate HSC collection numbers [Apperley et al., 2000].

The reduced transfusion requirements and hospital admissions for patients receiving HSC obtained from PB, as a result of faster recovery of neutrophils and platelets [Champlin et al., 2000, Vellenga et al., 2001] are some of the reasons that PB harvests are the preferred source of HSC [Gillespie and Hillyer, 1996, Nivison-Smith et al., 2005]. In addition, PB harvests are performed as outpatient procedures and the daily monitoring of $CD34^{+}$(a haematopoietic stem cell marker) numbers in the PB pre harvest can provide a reliable guide to HSC mobilisation prior to collection to ensure that harvests are performed when HSC rebound [Hass et al., 1994].

1.2 Indications

ASCT is increasingly considered as a treatment option for an expanding range of diseases including lymphomas, leukaemias, solid tumours and multiple myeloma.

1.2.1 Non-Hodgkins Lymphoma

Successful treatment of NHL with intensive chemotherapy and autologous HSC began in the 1970s [Appelbaum et al., 1978, Santos, 1983] and remains a treatment option for patients with chemotherapy-sensitive relapse of high or intermediate grade NHL and lymphoblastic lymphoma [Schmitz et al., 1996, Apperley et al., 2000, Vose et al., 2001]. A comparison study of ABMT with salvage chemotherapy, of 215 patients with relapses of NHL (intermediate and high grade) [Philip et al., 1995] reported event free survival at 5

years of 46% for recipients versus 12% for patients receiving chemotherapy alone (P=0.001) and an overall survival rate of 53% and 32% respectively (P=0.038). While ASCT survival data suggests a benefit for these patients in comparison to standard chemotherapy alone, the risks increase with patients over 60 years old [Chen et al., 2001]. Nevertheless, the ABMTRR reports that at eleven years post transplant, ASCT patients have a 39% survival probability compared with 37% for allogeneic related recipients [Nivison-Smith et al., 2005].

The effectiveness of the treatment of low grade NHL by ASCT remains uncertain [Apperley et al., 2000] as allogeneic transplants may offer a better chance of a cure as a graft versus low grade lymphoma effect may exist. A comparison study of 28 patients with refractory and recurrent low-grade NHL treated with allogeneic versus autologous transplantation [Verdonck et al., 1997] reported event-free survival rates at two years of 68% for allogeneic BMT patients and 22% for autologous BMT recipients.

1.2.2 Multiple Myeloma

ASCT for MM patients began in the late 1980s, following the observation that the myelotoxicity of high-dose melphalan, which has been standard treatment for MM for over thirty years, was reduced in patients following ABMT [Barlogie et al., 1986, Samson and Singer, 2001, Barlogie et al., 2004] Several comparison studies of ASCT and chemotherapy for treatment of MM have all reported improved event-free survival and overall survival of patients of approximately 52% at 5 years [Barlogie et al., 1997, Desikan et al., 2000, Lenhoff et al., 2000, Blade et al., 2003]. However, the presence of chromosome 13 abnormalities in some MM patients has been recognised as a poor prognostic feature [Jacobson et al., 2003] for ASCT outcome, with one report of 5 year event-free survival of 0% and overall to 16% [Desikan et al., 2000]. The ABMTRR reports that eleven years post transplant ASCT patients have a 25 % survival probability compared with 24% for allogeneic related recipients [Nivison-Smith et al., 2005].

1.2.3 Acute Myeloid Leukaemia

For AML patients over 45 years of age without an HLA matched donor, ASCT with intensive chemotherapy in first remission remains a treatment option [Champlin and Gale, 1987, Apperley et al., 2000]. As GVHD is not an issue, transplant morbidity and mortality for AML patients receiving autologous HSC transplants is reduced in comparison to allograft transplants,

however disease relapse remains a major cause of patient death following autologous transplantation [Burnett et al., 1998].

Comparison studies of allogeneic transplants and autologous transplants with intensive chemotherapy report average disease-free survivals ranging from 48% to 55% at three to seven years [McMillan et al., 1990, Zittoun et al., 1995, Burnett et al., 1998, Tsimberidou et al., 2003]. In addition, all these studies concluded that ASCT results in improved disease free survival in comparison to intensive chemotherapy. Furthermore, the Australasian Bone Marrow Transplant Recipient Registry (ABMTRR) reports that at eleven years post transplant, good prognosis ASCT patients have a 47% survival probability [Nivison-Smith et al., 2005].

1.2.4 Solid Tumours

ASCT also continues to be used in the treatment of solid tumours to replenish bone marrow which has been terminally damaged by high-dose chemotherapy used to destroy diseases such as neuroblastomas, glioma, soft-tissue sarcomas, germ cell tumours and Ewing's sarcoma [Apperley et al., 2000, Nivison-Smith et al., 2005, Gratwohl et al., 2004].

A 10 year European Blood and Marrow Transplantation (EBMT) study of 27902 HSCT reports a decline in use of ASCT for breast cancer, small cell lung cancer and ovarian cancers following an initial increase in transplant numbers up to 1997 [Gratwohl et al., 2004]. In the 1990s the most common solid tumour treated by ASCT was breast cancer, following the presentation of data at a meeting of the American Society of Clinical Oncology of a randomised trial of 154 patients [Bezwoda, 1999] demonstrating significant survival following high dose chemotherapy and ASCT. However a review of this study by a team of American investigators found serious inaccuracies and breaches of acceptable research practice in the study (Bearman, 2000; Ford, 2001). As a result, the findings have been revoked and ASCT is no longer promoted, as a treatment option for breast cancer patients as it does not appear to improve patient survival.

1.2.5 Other Diseases

ASCT may also be less commonly considered in the treatment of a number of diseases, including Chronic Myeloid Leukaemia (CML), Hodgkin's disease (HD), Amyloid light chain (AL) depending on patient age, disease status and availability of HLA-matched allogeneic donors [Apperley et al., 2000] [Gertz et al., 2004].

CML was the first disease for which ASCT was performed using HSCs collected by aphaeresis as large numbers of nucleated cells are present in the PB [Goldman et al., 1978]. In an attempt to retard the progress of the disease, albeit temporarily by restoring the chronic phase, ASCT may be of benefit for CML patients as HSCs collected while the patient is in chronic phase are transplanted when the patient progresses to blastic phase [Lemonnier et al., 1986, Cheson et al., 1989]. However as the transplanted cells are Ph+, the disease can not be cured by ASCT [Champlin and Gale, 1987]. ABMTRR reports that eleven years post transplant ASCT good risk patients have a 56% survival probability compared with 14% for poor risk ASCT patients [Nivison-Smith et al., 2005].

ASCT is considered as a treatment option for patient's with HD treated initially with chemotherapy who relapse, as well as HD patients with advanced HD treated with chemotherapy who do not enter complete remission [Apperley et al., 2000]. A three year comparison study of PB versus BM ASCT for 70 matched pairs of patients with HD treated with BEAM reported a overall survival of 68.6% for BMT and 78.2% for PBSCT and no major overall or progression-free survival advantage for PBSCT compared to ABMT [Perry et al., 1999]. A long-term follow-up study of 55 patients with relapsed or refractory HD treated with ASCT following Mini-Beam BCNU (carmustine, etoposide, cytarabine, melphalan) chemotherapy reported the overall seven year survival was 52%, the progression-free survival 54% and the event-free survival 36% [Martin et al., 2001]. Furthermore, the ABMTRR reports that at eleven years post transplant, ASCT HD patients ($n = 420$) have a 60% survival probability compared with 0 % for allogeneic related recipients ($n = 12$) [Nivison-Smith et al., 2005].

AL is a disorder of plasma cells resulting in the synthesis and deposition of insoluble immunoglobulin light chains in various organ systems causing progessive organ failure and death with a median survival of one year without ASCT [Benekli et al., 2000] [Worel et al., 2006]. Patient age, number of organs involved and extent of cardiac involvement are all strong indicators of patient survival following ASCT [Gertz et al., 2004]. However, AL patients generally report higher rates of morbidity and mortality following ASCT than other ASCT patients [Gertz et al., 2004] [Worel et al., 2006]. Nevertheless, ASCT can induce response rates of approximately 60%,with a recent study reporting 67% of patients (4/6) alive after a median follow-up of 39 months post ASCT [Worel et al., 2006].

1.3 Risks/Morbidity/Mortality of Autologous Transplantation

1.3.1 Recurrent Disease

While ASCT avoids the risks of GVHD associated with allogeneic transplants there remains the risk that the harvest will contain diseased cells [Champlin and Gale, 1987] and relapse is more common following autologous transplantation than allogeneic transplantation [Armitage, 1994, Schimmer et al., 2000]. Disease relapse was the cause of 52.7% of deaths in the first year after transplant for autologous transplants performed in Australia between 1991 and 2002. This figure rose to 79.6% as the cause of death in the second year post transplant [Nivison-Smith et al., 2005]. In addition, the graft versus leukaemia effect present in allogeneic transplants, associated with reduced relapse rates [Horowitz et al., 1990], is absent in autologous transplants, which may contribute to increased rates of relapse post transplantation when compared to allogeneic transplants [Zittoun et al., 1995].

With improved patient survival post ASCT, a small but significant risk of secondary cancers has been reported in patients treated for NHL and HD following use of chemotherapy and radiotherapy supported by ASCT [Witherspoon et al., 1989]. Post transplant therapy related myelodysplasia (t-MDS) and secondary therapy-related acute myeloid leukaemia (t-AML) have been reported as long term complications of ASCT [Mach-Pascual et al., 1998]. Prior chemotherapy with large doses of alkylating agents has been suggested as an important risk factor as have older age at time of diagnosis and transplant as well as exposure to previous radiotherapy, especially total body irradiation (TBI) prior to ASCT [Pedersen-Bjergaard et al., 2000, Metayer et al., 2003].

A retrospective study of 612 patients who had received ASCT for HD and NHL reported 22 cases of t-MDS/t-AML at a median of 1.9 years from transplant, with a 12.3 fold increased risk of developing t-AML with 11q23/21q22 abnormalities in patients who received VP-16 for HSC mobilisation [Krishnan et al., 2000]. Another study of 104 female patients receiving ASCT for NHL evaluated the predictive value of clonal BM haematopoiesis for the development of t-MDS/AML using an X-inactivation based clonality assay at the human androgen receptor locus (HUMARA). They concluded that a significant proportion of patients have clonal haematopoiesis at the time of ABMT and that clonal haematopoiesis, as detected by the HUMARA assay was predictive of the development of t-MDS/AML (P=0.004) [Mach-Pascual et al., 1998].

1.3.2 Neutropenia

One of the major indicators of successful HSC engraftment is the time taken for neutrophil numbers to recover to 0.5 and 1.0 $\times$ $10^9/L$ [Nivison-Smith et al., 2005]. During this time the patient is neutropenic and hence vulnerable to infections [Hoffman and Pettit, 1989]. The use of G-CSF post ASCT to promote HSC production has reduced the neutropenic phase post transplantation. In addition, the increasingly preferred use of PB HSC, and the associated improved recovery time for neutrophils [Damiani et al., 1997] continues to reduce the neutropenic phase and hospital stays [Vellenga et al., 2001, Nivison-Smith et al., 2005].

1.3.3 Sepsis

Although ASCT recipients are not affected by GVHD post transplant infections, they remain vulnerable to infection, especially during the neutropenic phase. Septicaemia accounts for 3.9% of deaths in the first year post ASCT in Australasia [Nivison-Smith et al., 2005] and as the majority of patients experience sepsis post transplantation, all ASCT patients are closely monitored. Most ASCT patients however, require hospitalisation for bacterial and fungal infections, which can be treated with broad-spectrum antibiotics and anti fungal agents [Willams et al., 1990].

1.3.4 Thrombocytopenia

The number of days taken for platelet numbers to reach 20 and 50 $\times$ 10^9/L are also used as indicators of the HSC engraftment [To et al., 1998]. Thrombocytopenia with bleeding, is a serious, although uncommon complication of ASCT and occurs mostly in patients refractory to platelet transfusions [Willams et al., 1990]. As with neutrophil recovery the use of G-CSF post transplant to promote maturation of HSC and the increasing use of PB as a source of HSC has reduced periods of thrombocytopenia [Champlin et al., 2000, Vellenga et al., 2001] and hence transfusion requirements.

1.3.5 Reinfusion Risks

The intravenous reinfusion of cryopreserved HSC has been associated with numerous side effects including: renal failure, nausea, vomiting, headaches, cardiac and neurological complications [Stroncek et al., 1991,

Styler et al., 1992, Okamoto et al., 1993, Limaye, 1997]. The causes and symptoms of reinfusion toxicity will be discussed in detail in section 1.6.

1.4 Stem Cell Processing and Assessment of Engraftment Potential

1.4.1 Bone Marrow Processing (Volume and RBC reduction)

As described in section 1.1.3, unprimed BM or BM primed by G-CSF is collected from the iliac crests, of patients under general anaesthetic by multiple aspirations, to which anticoagulant such as Acid Citrate Dextrose Anticoagulant (ACDA) or Heparin is added in controlled ratios to prevent clotting [To et al., 1998]. The BM harvest must also be filtered to remove fat, bone fragments and aggregates, either during collection or subsequent processing [Apperley et al., 2000]. The volume of cells removed from patients is determined by both the patient's weight and the nucleated cell count (NCC) per kg, which is calculated from the mid harvest full blood count. The recommendation of 3×10^8 NCC/kg is used to provide an indication of the adequacy of the harvest by using the number of nucleated cells as a guide to HSC numbers [Apperley et al., 2000]. This will be discussed in detail in section 1.4.2.

Following the completion of the aspirations, the BM harvest must be further processed by the use of centrifugation, to separate the cellular and plasma components. Generally a closed system, such as a cell washer or an aphaeresis cell separator, is used for this purpose in preference to a open system such as ficoll density separation [To et al., 1998]. The aim of this procedure is two fold; firstly to reduce the product volume by the removal of excess plasma and red cells, and secondly to isolate the buffy coat which contains concentrated mononuclear and hence HSCs, which is then ready for cryopreservation [Wells et al., 1979, To et al., 1998].

1.4.2 Peripheral Blood Processing

Collections of HSC from PB follow priming by G-CSF with/without chemotherapy, commence when the WCC rises post nadir to 1.0×10^9/L, at which point most institutions begin CD34 testing. When $CD34^+$HSC numbers reach the institution's prescribed starting point ($5–10 \times 10^6$/ml), collection of HSC using mononuclear aphaeresis begins [Boiron et al., 1996]. PB collections are performed using continuous-flow cell separators to process, on average, 10

litres of blood but this is dependent on the patients needs [Apperley et al., 2000]. As red blood cells and plasma are returned to the patient throughout the process, the ultimate product of the aphaeresis is a buffy coat containing mononuclear cells suspended in plasma, which is then ready for cryopreservation.

1.4.3 Cryopreservation

Cryopreservation of HSCs evolved from animal [Mannick et al., 1960, Thomas and Ferrebee, 1962, Cavins et al., 1962, Buckner et al., 1970] and human studies [Malinin et al., 1970, Rybka et al., 1980] as a result of the need for long-term maintenance of HSC viability and function until the ASCT was performed. Cryoprotectant agents and the effect of storage on HSC function will be discussed in detail in section 1.5.

Following the addition of the cryoprotectant solution to HSCs, the suspensions are then cryopreserved in a controlled rate freezer with a cooling rate of 1 °C–3 °C per min to at least −40 °C. Upon completion of the freezing run the HSC are transferred to long term storage using liquid or vapour phase nitrogen at a temperature below −120 °C [To et al., 1998].

1.4.4 Assessment of Engraftment Potential

As HSC can not be identified by cell morphology, other methods have evolved to assess the engraftment potential of HSC harvests [Audet et al., 1998]. The earliest method of assessing engraftment potential was simply to monitor the recovery of haematological function of lethally irradiated subjects (both animal and human) post ASCT [Buckner et al., 1970, Malinin et al., 1970] and it still remains the ultimate test [Rybka et al., 1980].

The NCC per kg of patient weight of HSC harvests (i.e. cell dose) has been used as a indicator of neutrophil and platelet recovery [Stewart et al., 1989]. However, as NCC includes various nucleated cells including mature neutrophils, which do not survive freezing and thawing [Rybka et al., 1980], the validity of NCC as a indicator of engraftment potential has been questioned [al Fiar et al., 1997]. These findings are supported by an earlier study which also concluded that the dose of mononuclear cells infused did not correlate with neutrophil and platelet recovery [van der Wall et al., 1994]. Furthermore, Rowley et al 1994 reported that the cellular concentration of the cryopreserved cells (i.e.cells per ml) prior to freezing is also not predictive of post-thaw recovery of nucleated cells (P=0.38), granulocyte-macrophage colony-forming unit (GM-CFU)(P=0.06) or $CD34^{+}$cells (P=0.54), or for the

viability of mononuclear cells (P=0.81). Additionally, the cellular concentration of the cryopreserved product did not predict the time of neutrophil and platelet recovery post ASCT [Rowley et al., 1994].

The need for a in vitro assay to assess HSC collections was clearly identified and so progenitor cell assays for neutrophils, macrophages and erythrocytes evolved [Appelbaum et al., 1978, Apperley et al., 2000]. The Colony Forming Units Granulocyte Macrophage (CFU-GM) assay is routinely performed to assess the ability of the HSCs to engraft and mature into macrophages and granulocytes, an indication of the haematopoietic progenitor content of a stem cell collection [Eaves and Lambie, 1995]. CFU-GM assays do not provide information on primitive progenitors or progenitor cells committed to other lineages and are consequently an indirect test of engraftment potential [Appelbaum et al., 1978, Rybka et al., 1980, To et al., 1997]. Correlation of neutrophil and platelet recovery with CFU-GM numbers has been demonstrated by numerous studies [Spitzer et al., 1980, Rybka et al., 1980, Lemonnier et al., 1986, Rowley et al., 1994, van der Wall et al., 1994].

Although the CFU-GM assay provides valuable information regarding the proliferative potential of HSC collections it has several limitations. The 14 day incubation period required for CFU-GM assays ensures that this method can not be used for rapid assessment of HSC numbers prior to PB collection [Hass et al., 1994]. In addition, CFU-GM assays have exhibited poor reproducibility of results and therefore are being increasingly replaced by $CD34^+$analysis [Chang and Ma, 1998, Rock et al., 2000].

The enumeration of cells expressing $CD34^+$antigens is the gold standard for assessment of stem cell collections engraftment potential, as it provides a rapid indication of HSC numbers [Siena et al., 1991, Keeney et al., 1998]. As CD34 analysis can be performed in approximately 1 hour, it is the most suitable method of monitoring HSCs during PB mobilisation, to allow optimisation of aphaeresis collections [Hass et al., 1994, Keeney et al., 1998]. Correlation of CD34 numbers with platelet and neutrophil recovery has been demonstrated by numerous studies [Rowley et al., 1994, van der Wall et al., 1994, Keeney et al., 1998, Apperley et al., 2000]. $CD34^+$assay results of HSC may not always correlate with CFU-GM assay results as the CD34 antigen is found on both HSC and committed progenitors, with only a portion of the $CD34^+$cells capable of triggering and sustaining long-term haematopoiesis. The CD34 antigen is believed to be involved in cell-cell interactions or cell-matrix adhesions [Chang and Ma, 1998].

The adoption of International Society of Hematotherapy and Graft Engineering (ISHAGE) guidelines for detection of $CD34^+$cells based on a

four parameter flow cytometry method (CD45FITC/CD34 PE staining, side and forward angle light scatter) [Keeney et al., 1998] by most institutions should increasingly counter the lack of inter-institutional reproducibility [Chang and Ma, 1998, Rock et al., 2000]. The modification of the ISHAGE guidelines by the addition of a known number of fluorescent microspheres, removes the need for a WCC from a automated haematology analyser consequently removing another source of potential error, while converting the flow cytometer into a single platform $CD34^+$cell-counting device [Keeney et al., 1998]. The incorporation of viability dyes such as 7AAD into single platform analysis allows the determination of the absolute viable $CD34^+$cell count which can then be used for post cryopreservation sample analysis [Keeney et al., 1998].

Currently 1.5–2.0 $\times$ 10^5 CFU-GM/kg or 1–2 $\times$ 10^6 $CD34^+$cells/kg are generally agreed as the minimum threshold below which haematopoietic reconstitution will not occur [To et al., 1997].

1.5 Cryobiology

1.5.1 Cryoprotective Agents

Cryopreservation of HSC requires the addition of a cryoprotectant to preserve cell viability and function during storage at ultra low temperatures for variable lengths of time [Apperley et al., 2000]. Without a suitable cryoprotectant the formation of intra-cellular ice may rupture HSCs and extra-cellular ice formation will result in the dehydration of HSCs as water is taken up by the growing ice crystals [Rowley and Anderson, 1993]. Cryoprotectants including glycerol, hydroxylethyl starch (HES) and dimethyl sulfoxide (DMSO) have all been used in the cryopreservation of HSC [Rowley and Anderson, 1993].

Although there are small variations in technique between institutions currently the majority of institutions use a mixture of 5–15% DMSO and 1–25%(v/v) protein supplement (autologous plasma or human serum albumin), which is added to the HSC so that nucleated cell concentration (NCC) remains below 3 $\times$ 10^8/ml [To et al., 1998, Apperley et al., 2000]. Samples of HSC suspension are removed for microbial contamination testing prior to cryopreservation [To et al., 1998].

DMSO, a by product of the paper industry, is the principal cryoprotectant used in the cryopreservation of haematopoietic stem cells (HSC) intended for autologous transplantation after high dose chemotherapy [Rowley et al., 1999a]. DMSO, a dipolar, aprotic solvent acts as a pen-

etrating agent, protecting HSC from ice crystallisation on a molecular basis by reducing the electrolyte concentration in the residual unfrozen solution [Rowley, 1992]. The hydrogen bonding between DMSO and water is approximately 1.3 times stronger than between water and water [Brayton, 1986] although these properties are only exhibited within a certain concentration range [Swanson, 1985]. The optimum concentration of DMSO appears to be between 5 and 10% of the final frozen volume [Apperley et al., 2000].

The effectiveness of 10% DMSO as a cryoprotectant in animals studies [Cavins et al., 1962, Malinin et al., 1970, Swanson, 1985] lead to the acceptance of DMSO in the cryopreservation of human HSC [Buckner et al., 1970]. However, DMSO poorly preserves granulocytes and erythrocytes as the osmotic tolerance of these cells is much less than lymphocytes and HSC [Brayton, 1986, Davis et al., 1990, Rowley, 1992]. Consequently, the reinfusion of cryopreserved HSCs has been associated with DMSO related toxicities, which will be discussed in greater detail in section 1.6 [Rowley and Anderson, 1993].

1.5.2 Effect of Storage on HSC Function (Temperature, Time)

ASCT requires the storage of HSC at ultra low temperatures for extended periods and the effect of storage on HSC function has been of concern since the 1960s and continues to be in the present day [Mannick et al., 1960, Cavins et al., 1962, Lewis and Trobaugh, 1964, Malinin et al., 1970, Buckner et al., 1970, Rybka et al., 1980, Rowley et al., 1994]. The effect of both storage temperature and length of storage have been assessed by numerous studies using CFU-GM assays and increasingly CD34 enumeration.

The most effective storage conditions for HSC for prolonged periods is in the liquid phase of nitrogen, $-196\,^{\circ}\mathrm{C}$, as vapour phase storage is subject to temperature fluctuations [Rybka et al., 1980]. A comparison study of HSC stored in liquid nitrogen with dry ice ($-78.5\,^{\circ}\mathrm{C}$) storage after 3 years [Malinin et al., 1970] reported that cells stored in liquid nitrogen were largely intact while those stored in dry ice were essentially destroyed. Storage of HSC at low temperatures (i.e. $-196\,^{\circ}\mathrm{C}$) should result in a complete and homogenous blockade of all enzymatic pathways, which should limit any deterioration in HSC viability [Aird et al., 1992]. A controlled retrospective study of the engraftment data of 33 ASCT patients whose HSC had been stored for 2 years or more in liquid nitrogen reported that HSC stored for up to 11 years was capable of engraftment [Aird et al., 1992].

A study into the effects of background radiation on Chinese ham-

ster ovary fibroblasts cryopreserved in 10% DMSO and stored at −196 °C estimated that a time period of approximately 30,000 years was required to cause cellular destruction in HSC stored under these conditions [Ashwood-Smith and Friedmann, 1979]. Consequently, as ASCT are only required for the life-span of the human recipient it seems unlikely that this would be an issue.

Investigation of cryopreservation of cord blood HSC cells by CFU-GM, BFU-E and CFU-GEMM assessed after 6 months, 2 and 12 years storage in liquid nitrogen reported no significant loss of HSC function [Mugishima et al., 1999]. This study is supported by a retrospective study [Attarian et al., 1996] which found no detectable deterioration of cells stored for up to 7.8 years. These findings suggest that HSC may be stored for prolonged periods at ultra low temperatures without adversely affecting the engraftment potential of HSC.

1.6 Reinfusion associated toxicity

1.6.1 Causes (DMSO, RBC's, vol. Overload, histamine)

Cryopreserved HSC are usually thawed at or near the patient's bedside in a 37 °C waterbath and the cells are then reinfused as quickly as possible into the patient [Bostrom and Burger, 1999]. Hence DMSO is routinely reinfused intravenously along with the thawed cell products [Egorin et al., 1998] and is associated with a number of dose dependent DMSO infusion related toxicities [Stroncek et al., 1991]. Many of these effects are due to DMSO induced histamine release by mast cells [Brayton, 1986]. The frequency of ASCT side effects correlates with the volume of infused cryopreserved cells, the dose of DMSO and the volume of cell lysis products infused [Styler et al., 1992, Okamoto et al., 1993].

Red blood cells lyse when frozen and thawed, resulting in renal tubule damage, pyrexia and hyperkalemia [Okamoto et al., 1993]. These side effects stimulate the endothelial cells to produce thromboplastin, which activates coagulation factors and induces disseminated intravascular coagulation (DIC), further increasing damage to kidneys [Okamoto et al., 1993]. In addition, complement activation and mast cell de-granulation results in the production of histamine, leukotriene and anaphylotoxin, which can cause transient shock [Bostrom and Burger, 1999].

The presence of damaged granulocytes in cryopreserved cell solutions can lead to cell clumping upon thawing, which has the potential to decrease the number of HSCs being reinfused into the patient, which in turn could

affect patient recovery from ASCT. The reinfusion of histamine released by the dead and damaged granulocytes can trigger vasodilatation of local blood vessels and smooth muscle contractions, leading to demonstration of some of the symptoms of immediate hypersensitivity reactions [Janeway et al., 1999].

Fluid overload as a consequence of the reinfusion of large volumes of cryopreserved cells into patients is also a potential cause of reinfusion related toxicity especially for patients with renal and cardiopulmonary insufficiency [Okamoto et al., 1993, Dhodapkar et al., 1994].

1.6.2 Symptoms

ASTC patients experience reinfusion related symptoms more than allogeneic HSC patients and these toxicities are largely related to DMSO [Stroncek et al., 1991, Lopez-Jimenez et al., 1994, Bostrom and Burger, 1999]. Nausea and vomiting are the most commonly reported side effects of ASCT [Davis et al., 1990]. However numerous other ASCT related symptoms have been reported with varying frequency including: headache, fever, abdominal cramping, flushing, decreased heart rate, neurological toxicity and increased blood pressure [Davis et al., 1990, Stroncek et al., 1991, Rowley et al., 1999b, Hoyt et al., 2000].

DMSO is excreted primarily via urine as dimethyl sulfone (DMSO2) with less than 2% excreted by urea and 3% is exhaled as a metabolite, dimethyl sulfide (DMS) which is associated with a unpleasant "garlicky" odour [Brayton, 1986]. A study of 22 oncology nurses treating recipients of ASCT reported that 91% of nurses had personally experienced symptoms such as headache and nausea following exposure to DMS. As a consequence they attempted to avoid odour contact and hence potentially compromised patient treatment [Prior et al., 2000]. As the estimated half life of DMSO in man is approximately 11 to 14 hours and the half life of DMS is approximately 24 hours [Swanson, 1985] ASCT patients, their family members and hospital staff could be exposed to DMS side effects for a extended period of time.

Kessinger etal (1990) found that of 100 ASCT patients, 92% experienced haemoglobinuria and 80% experienced red urine post reinfusion of cryopreserved HSC [Kessinger et al., 1990]. Acute renal failure, heart attack or cardiac arrest have also been reported as a result of the contamination of red blood cell lysates and DMSO [Styler et al., 1992, Rowley et al., 1999a]. The release of histamine by DMSO or from damaged granulocytes has been reported to cause various degrees of AV nodal conduction block [Styler et al., 1992]. Additional factors such as the mechanical effect of large volume reinfusion, cell lysis products such as potas-

sium, calcium or adenosine, and hypothermia of the infused HSCs has also been suggested as possible cause of bradyarrhythmia in ASCT patients [Styler et al., 1992, Keung et al., 1994, Lopez-Jimenez et al., 1994].

1.6.3 Treatment

The pre-medication administration of antihistamines prior to reinfusion reduces the ASCT symptoms related to histamine release [Davis et al., 1990] and most symptoms resolve over a few hours and are treated symptomatically [Rowley et al., 1999a]. However those ASCT patients with renal insufficiency may require plasmapheresis post reinfusion to reduce DMSO related side effects if a large volume of HSC is reinfused [Dhodapkar et al., 1994].

Correlation between the volume of transplanted cells and hence the volume of DMSO and side effects has been demonstrated by several studies [Davis et al., 1990, Kessinger et al., 1990, Dhodapkar et al., 1994]. The maximum dose of DMSO which can be safely reinfused during ASCT is generally considered to be 1g DMSO/Kg/day [Bostrom and Burger, 1999]. In the event that a large volume of cryopreserved cells needs to be reinfused the reinfusion should be conducted over two days to limit patient DMSO exposure [Bostrom and Burger, 1999].

Currently there are two main strategies for reducing ASCT related toxicities. Firstly the total volume of cells cryopreserved can be reduced and thus the volume of DMSO and cell lysis products for reinfusion will be reduced [Davis et al., 1990]. Increasing the NCC during cryopreservation to 5×10^8/ml has accordingly been suggested as a means of reducing DMSO requirements [Rowley et al., 1994]. Secondly, DMSO and the cell lysis products can be removed by washing post-thawing consequently removing the suspected cause of ASCT related toxicities [Beaujean et al., 1991, Syme et al., 2004].

1.7 Removal of Toxins prior to Transplant

1.7.1 Concerns

Previous studies have expressed concern that the removal of DMSO by washing may reduce the viability and/or number of HSC available for reinfusion and so adversely affect the engraftment potential of an ASCT. Ragab et al 1977 reported the nucleated cell recovery of cryopreserved samples before washing was approximately 100%, while post washing the recovery was only between 30 to 50%, concluding that post-thaw washing was detrimental to HSC [Ragab et al., 1977]. These findings were supported by Broxmeyer et

al 1989 who reported an 80-100% recovery of cryopreserved nucleated cells before washing and concluded that any washing of cells to remove DMSO severely decreased the recovery rate of nucleated cells [Broxmeyer et al., 1989]. However Rubinstein et al 1995 have reported improved recovery of viable cryopreserved umbilical cord blood HSC following washing with isotonic solution [Rubinstein et al., 1995].

1.7.2 Washing protocols

The New York Cord Blood Bank (NYCBB) washing protocol as reported by Rubinstein et al 1995 concluded that DMSO could be removed from cryopreserved cord blood by washing thawed cryopreserved HSC without affecting the cell viability or clonogenic ability [Rubinstein et al., 1995]. Immediately after thawing, an equal volume of isotonic albumin/dextran diluent solution is slowly added with continuous mixing before centrifugation at 400g for 10 mins. The supernatant is then removed and the cell pellet slowly resuspended in fresh albumin/dextran solution to the original volume.

1.8 Clinical Trial

Although predominately used for UCB, post-thaw stem cell washing is an increasingly common laboratory practise prior to ASCT [Rubinstein et al., 1995, Antonenas et al., 2001, Syme et al., 2004, Lemarie et al., 2005]. However this procedure needs to be balanced against the fact that morbidity has already been significantly lowered by reductions in the reinfusion volume (achieved through refined harvesting, mobilising and processing techniques) and improved patient pre-medication. A formal clinical trial is required to properly assess whether such a the change in procedure significantly benefits HSC recipients.

1.8.1 Quality of Life Assessment

Analysis of morbidity can be assessed qualitatively through the use of quality of life assessments (QOL). Health-related QOL refers to the extent which one's usual or expected physical, emotional and social well-being are affected by a medical condition or it's treatment [Cella, 1996]. Since the 1970s QOL has become central to oncology research as opposed to simply cure focussed research [Aaronson, 1990, Cella and Tulsky, 1993]. This reflects an understanding that a severely toxic treatment must have its

harmful side effects weighed against its ultimate impact on survival prognosis [Cella and Bonomi, 1995]. This information can then be used to evaluate the efficacy of competing medical interventions and as a guide to improving care delivery [Cella, 1996]. QOL is now so highly valued that the US Federal Drug Administration (FDA) has stated that benefit to QOL is one of two possible reasons for approval of new anticancer drugs, with improved survival being the other criteria [Cella, 1996].

1.8.2 Patient and Test Selection

As the patient is the most authoritative source of QOL information, such surveys are subjective and the selection of subjects should be guided by statistical considerations [Aaronson, 1990] . A major consideration is the calculation of required sample size. This may be determined by a combination of three factors: alpha level (usually set at $P < 0.05$); power, or the probability that the study will accurately confirm the hypothesis; and effect size, the extent to which the phenomena under study truly differs amongst samples [Cella and Bonomi, 1995].

In addition to statistical considerations, the use of pilot studies will establish the suitability of questions for patients as well as any potential problems with access. Any practical concerns can then be addressed before commencing a clinical trial. Preliminary investigations may also help to clearly identify the population of interest and hence refine the inclusion/exclusion criteria of a QOL survey on the basis of relevant criteria (such as disease, treatment, age, gender, ...) [Cella and Tulsky, 1993].

The most common issues reported in QOL surveys in oncology are pain, nausea and fatigue [Cella and Tulsky, 1993]. As QOL measurements are subjective there is no 'gold standard' and the numerous QOL surveys available can be grouped into three broad categories [Cella, 1996].

1. Descriptive QOL information may be obtained from the use of open ended questions during interviews such as the Perceptual QOL, developed for the study of cancer patients following radiation therapy.

2. Multiple choice or analogue (scaled) QOL data responses may also be acquired used in surveys such as the Linear Analog Self Assessment (LASA) developed for the general cancer patient population.

3. A combination of both styles may be employed such as the Psychosocial Adjustment to Illness Scale (PAIS) for general illness [Cella and Tulsky, 1990].

Furthermore, in order reduce problems associated with non compliance, the potential burden to patients or staff must be a considered as well as

the ability of a particular QOL scale to meet the needs of the investigator [Cella, 1996]. An existing scale can be modified to include relevant and specific items not included in the scale in order to evaluate additional criteria [Cella and Bonomi, 1995]. QOL surveys can be rated by an observer such as a health care professional or family member, however these observations do not always agree strongly with patient self ratings and therefore are considered to be less reliable [Cella and Tulsky, 1993].

1.8.3 Quality of Life Surveys and Bone Marrow Transplantation

The practitioner must weigh the benefits of the transplant versus the patients quality of life [Cella and Webster, 1999]. Consequently the concept of QOL is of interest to the area of ASCT especially in the instances where the procedure outcome may not always be guaranteed (as in the case of treatment of low grade lymphoma). Furthermore, QOL surveys have also been used to evaluate the impact of BMT in order to improve quality of patient care.

A qualitative study of 119 AlloBMT patients consisting of a one-time interview using six open-ended questions. [Ferrel et al., 1992] demonstrated that four dimensions of patient well-being were affected:

Physical: infertility, nutrition, functional activity, strength and stamina, visual disturbances/cataracts, recurrent colds, coping with chronic GVHD;

Psychological: anxiety, second chance, depression, fear of recurrence, changed priorities, cognition/attention, coping with survival;

Spiritual: hope, despair, religiousity, inner strength and

Social: appearance, leisure activities, return to work, financial burden, roles and relationships, affection/sexual function.

Further analysis of patient responses identified factors capable of influencing their QOL both positively (such as good health, family and friends, appreciating life, ...) and negatively (such as unfulfilled goals, losing relationships, financial distress, ...). Moreover, patients identified areas where health professionals could enhance QOL including; being accessible, providing support groups and increasing patient participation in decision making [Ferrell et al., 1992].

Studies of AutoBMT survivors based on the QOL methodology of [Ferrel et al., 1992] have found that most AutoBMT patients experienced few long term side-effects and enjoyed above average QOL that was

equal to and sometimes superior to pre AutoBMT [Whedon et al., 1995, Saleh and Brockopp, 2001] . However, areas of AutoBMT patient concern included fear of recurrence, fear of secondary cancer and sexual function as also identified by AlloBMT patients [Ferrel et al., 1992]. Nevertheless, a multicentre questionnaire study of 200 adult allogeneic (46%) and autologous (54%) BMT patients at least 12 months post transplant, concluded that in general AlloBMT patients reported poorer QOL [Andrykowski et al., 1995]. The use of pre- and post-BMT QOL assessments of physical, psychological and spiritual functioning would identify those patients requiring specialised assistance [Whedon et al., 1995, Saleh and Brockopp, 2001].

Investigation of the impact of BMT on QOL of patients has lead to improved understanding and identification of patient needs. However, a study of 150 oncology nurses using a mailed Quality of Life-BMT Survey (used previously with patient analysing perceptions of the meaning of QOL for BMT patients), revealed that nursing staff believed that patients had a lower QOL than the patients themselves believed [King et al., 1995]. The failure to adequately assess patient needs could result in a focus on physical aspects rather than incorporating a more holistic approach.

1.8.4 Prior Studies of Quality of Life and Reinfusion Related Side-effects

The impact of the post-thaw removal of DMSO for patients undergoing ASCT has been previously evaluated by use of a visual analog scale QOL (VAS-QOL) questionnaire measurement of infusion related symptoms [Rowley et al., 1999a]. The aim of that study was to determine if in addition to preventing dose related DMSO toxicites, if the NYCBB washing protocol would remove the need for anti-histamine pre medication (such as diphenhydramine).

Using a modified NYCBB washing protocol, cryopreserved cells were washed with 10% dextran-40 and 5% Human Serum Albumin (HSA) solution, the supernatant expressed and then transported to the patients bedside for immediate reinfusion. Five patients enrolled in the study completed two surveys, one immediately before the reinfusion and the other post reinfusion. The patients enrolled in the study did not receive any medication within 2 hours preceding the ASCT. Furthermore, nursing staff attending to the patient also documented an assessment of patient toxicity and vital signs before, during and after completion of the infusion.

However, this study was abandoned for safety reasons after one patient experienced severe infusion-related toxicity. Conse-

quently, [Rowley et al., 1999a] acknowledged that anti-histamine medication should be part of ASCT patient care, however they recommended pre freezing volume reduction to reduce the quantity of cells stored and hence the amount of DMSO used as a more efficacious approach to reducing infusion-related toxicity.

1.9 Summary

There have been numerous developments in ASCT since its discovery as a consequence of the post world war two investigations into the effects of irradiation. Currently the number of autologous transplant procedures performed exceeds allogeneic procedures as increasing applications are found for this procedure. However, as HSCs require storage for variable lengths of time before ASCT, cryopreservation is necessary in order to maintain cellular viability and function during storage of at ultra low temperatures [Apperley et al., 2000]. DMSO, a by product of the paper industry, is the principal cryoprotectant used in the cryopreservation of HSC and hence is routinely reinfused intravenously along with the thawed cell products [Egorin et al., 1998]. The intravenous reinfusion of cryopreserved HSC has been associated with numerous side effects including: renal failure, nausea, vomiting, headaches, cardiac and neurological complications [Stroncek et al., 1991, Styler et al., 1992, Okamoto et al., 1993, Limaye, 1997]. The frequency of ASCT side effects correlates with the volume of infused cryopreserved cells, the dose of DMSO and the volume of cell lysis products infused [Styler et al., 1992, Okamoto et al., 1993].

Currently there are two main strategies for reducing ASCT related toxicities. Firstly, the total volume of cells cryopreserved can be reduced and thus the volume of DMSO and cell lysis products for reinfusion will be reduced [Davis et al., 1990]. Secondly, DMSO and the cell lysis products can be removed by washing post-thawing consequently removing the suspected cause of ASCT related toxicities [Beaujean et al., 1991, Syme et al., 2004].

The utilisation of post-thaw stem cell washing is an increasingly common practise [Beaujean et al., 1991, Rubinstein et al., 1995, Antonenas et al., 2001, Syme et al., 2004], however this needs to be balanced against the fact that morbidity has already been significantly lowered by reductions in the reinfusion volume (achieved through refined harvesting, mobilising and processing techniques and improved patient pre-medication). The question this thesis will seek to answer is: does any reduction in patient morbidity from washing warrant use of this technique in the presence of reduced reinfusion volumes of DMSO? Evaluation of ASCT patient well-being

through the use of QOL surveys may provide a valuable assessment of the impact of post-thaw stem cell washing on patient morbidity. This information can then be used to evaluate the efficacy of competing medical interventions and as a guide to improving care delivery [Cella, 1996].

Project Aims

This thesis reports on a validation study of a modified version of New York Cord Blood Bank's (NYCBB) washing protocol (Rubinstein et al. 1995) in which the rate of centrifugation was increased to 1400g. The aim of this modification was to improve the separation of the HSC pellet from the supernatant to reduce cell loss during plasma expression. Increasing centrifuge speed will increase the sedimentation rate and the density of the cell pellet, raising the spectre of damage to HSC because of this adjustment. Therefore the principal aim of the laboratory study was to carefully and thoroughly validate the impact of the washing procedure on the efficacy and safety of HSC transplantation. This was accomplished through multi-parameter analysis of the cell recovery [Hartmann et al., 1985] and engraftment potential [Spitzer et al., 1980, Chang and Ma, 1998] of washed and unwashed samples.

This thesis also presents findings of a clinical trial using a modified version of the VAS-QOL surveys [Rowley et al., 1999a] in which all patients enrolled in our non-randomised trial received pre medication prior to ASCT. The primary objective of this study is to determine whether washing of thawed HSC products is clinically justified. This is achieved by measuring the morbidity experienced by patients who receive *Washed* HSC as well as those patients who receive *Unwashed* HSC. Patients enrolled in this study completed four VAS-QOL surveys at pre-determined intervals within 24 hours of ASCT.

In addition, as the majority of reinfusions are performed in the outpatient setting where care of the patient by family members supplements nursing staff, we studied the impact of the washing protocol on the side effects experienced by nurses and carers. If this study validates the efficacy of the washing procedure in a clinical setting, then it will potentially lead to improved patient comfort (and collaterally nursing and family members) through the safe removal of toxic compounds in stem cell harvests.

Chapter 2

Laboratory Validation

2.1 Introduction

The aim of this laboratory validation study was to investigate if toxic DMSO and cell lysis products can be safely washed out from thawed stem cell harvests without affecting stem cell viability and engraftment potential using standardised and reproducible multiple parameter testing. Cellular pellets spun at 400g were found to be relatively "soft" and thus were prone to dislodging during plasma expression. Therefore the centrifuge speed was increased to 1400g to harden the pellet and reduce the risk of cell loss during plasma expression.

The results of the multiple parameter testing will provide an accurate record of the impact of the washing protocol on HSC viability and engraftment potential. In addition, the impact of the washing protocol on free haemoglobin levels will indicate its effectiveness in removing cellular lysis products, and by association, DMSO. This modified HSC washing protocol will demonstrate whether this is a feasible clinical practice that has the potential to reduce the morbidity associated with the reinfusion of cryopreserved stem cells.

2.2 Materials and Methods

2.2.1 Maintenance of Sterility

Sterility was maintained with a Class II Biological Safety Cabinet where required.

Table 2.1: Reagents and Suppliers

Reagents	Suppliers
0.9 % Saline Infusion BP AHB 1323	Baxter
7-AAD	Sigma Chemical Company
ACD-A AHB7898	Baxter Fenwal
ProCOUNT 340498	Becton Dickinson
20% Human Serum Albumin (HSA)	CSL Ltd
Gentran 40 Dextran 40 I.V Infusion	Baxter
BP 10% w/v in NaCl I.V. Infusion	
BP 0.9 w/v B5043	
Methocult(tm)[1]GF 4534	AMRAD Pharmacia
(Batches HC8LO5TO37 and HC9B19RO38)	
Monoclonal antibodies[2]	
Anti-HPCA-2 CD34 PE 348057	Becton Dickinson
Anti-HLe-1 CD45 FITC 347463	Becton Dickinson
Isotype Control: Mouse IgG1 PE 349043	Becton Dickinson
PBS tablets BR0014G	Oxoid
Optilyse B 1400	Immunotech
Trypan Blue (0.4% solution, PO4 buffered NaCl) 16-910-49	ICN

2.2.2 Solutions

Isotonic Diluent Solution

Gentran	500 ml
Saline	400 ml
HSA	100 ml

Components were mixed and stored at 4 °C.

PBS

10 PBS tablets were dissolved in de-ionised distilled water and made up to 1000ml with distilled water. PBS solutions were filtered and stored at 4 °C.

[1]Methocult batches underwent testing for batch-to-batch variability as part of the Stem Cell Transplant Laboratory, Royal Hobart Hospital, routine quality control.

[2]All antibodies used had undergone verification of reactivity as part of routine Quality Assurance in the Flow Cytometry Laboratory, Royal Hobart Hospital.

Table 2.2: Disposables and Manufacturers

Disposables	Manufacturers
10 ml syringe with Luer lock SS+10L	Terumo
18 G Drawing up needle, non-bevelled 04038088	Braun
30ml sterile V bottom containers 10425003	Techno-Plas
3ml syringe with Luer lock 302113	Becton Dickinson
60 ml syringe with Luer lock SS60LA	Terumo
600ml Fenwal Transfer pack container with coupler 4R2023	Baxter
Coupler with female Luer 912-647-904	Cobe
Falcon tube with cap (sterile) 352058	Imbros Pty Ltd
Hemastix 2816	Bayer
Mixing Cannulae 500.11.012	Indoplas
Petri dish (35 x 10 mm with 2 mm grid) 174926	Corning

Table 2.3: Equipment and Manufacturers

Equipment	Manufacturers
4 well plate	Corning
400 Cyclic Fridge	Kelvinator
Class II Biological Safety Cabinet	Gelman Sciences Australia
FACScan Flow Cytometer	Becton Dickinson
Haemocytometer ($0.1mm^2$)	Superior
Incubator (5% CO_2)	Heraeus Instruments
Inverted Microscope	Olympus
Microfuge	Denver Instruments
PB 3000 Scales	Mettler
Plasma Extractor	Fenwal
RF Sealer Model 2101	Sebra
Varifuge 3.0RS	Heraeus Instruments
Vortex mixer	Ratek Instruments
Waterbath	Ratek Instruments

Stock 7AAD Solution

7AAD	1 mg
DMSO	0.5 ml
PBS	0.5 ml

A stock solution was prepared by dissolving the 7AAD in DMSO and then in PBS. To make the working solution, $975\mu L$ of PBS was added to $25\mu L$ of 7AAD stock solution (final concentration 25g/ml), which was then aliquoted into tubes. One tube of the working solution was stored at 4 °C in the dark, and the remainder of the aliquots were stored at −20 °C until required.

2.2.3 Study Samples

Ethical Approval

Approval for this study was obtained from the University of Tasmania Human Research Ethics Committee (Project No. 9844).

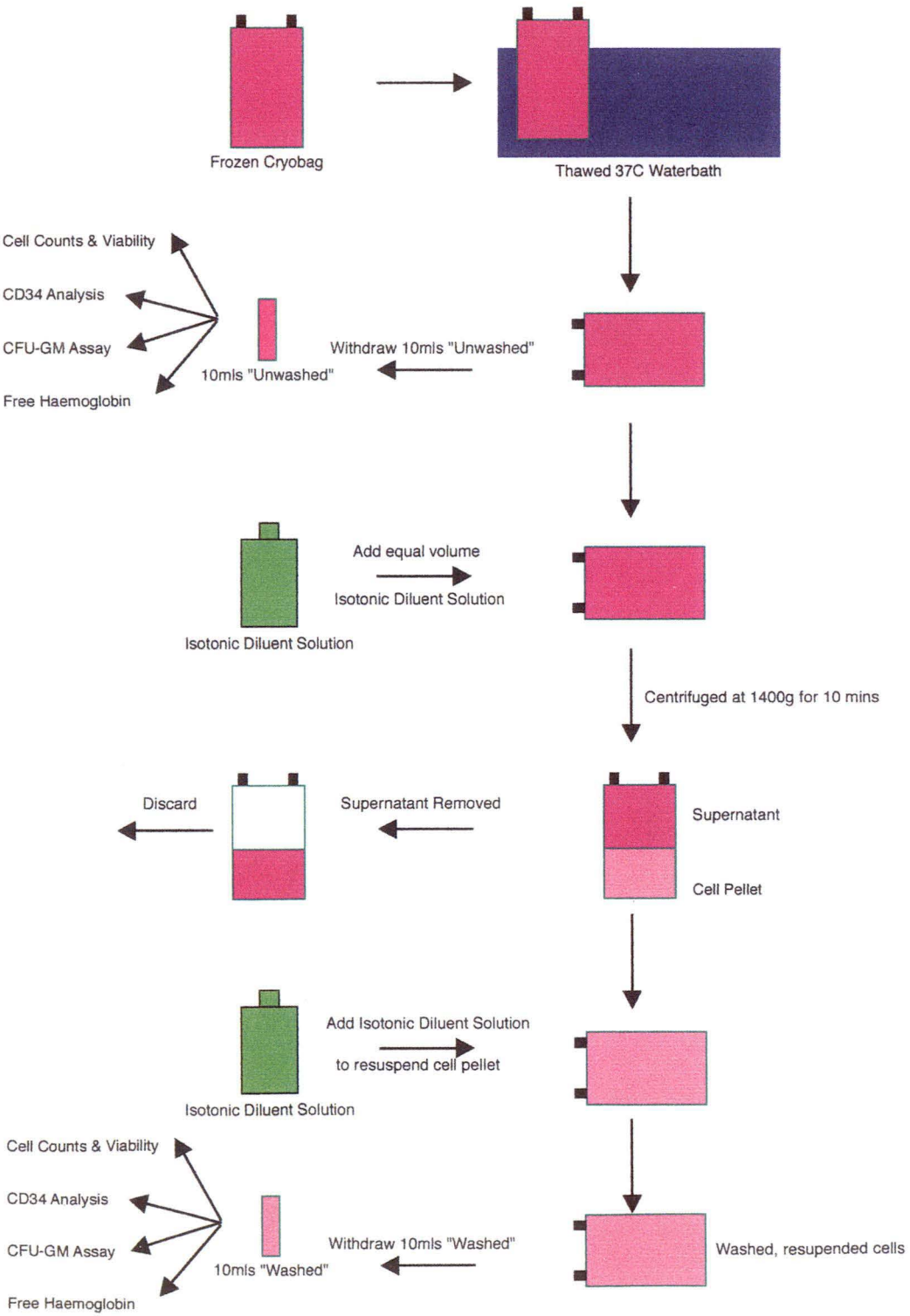
Selection of the study samples

Fourteen frozen cryobags containing stem cell collections from deceased patients (seven bone marrow harvests and seven peripheral blood harvests) were used for this laboratory validation study. All study samples had been harvested and processed at the RHH between 1997 and 1998 and stored in the liquid phase of liquid nitrogen until transfer to storage in the Stem Cell Laboratory −80 °C freezer immediately prior to the time of this study.

2.2.4 Stem Cell Washing Protocol

Fourteen frozen cryobags containing stem cell collections from deceased patients (seven bone marrow harvests and seven peripheral blood harvests) were rapidly thawed in a waterbath at 37 °C. Using aseptic techniques, the bags were spiked with a blood pack adaptor and a 10ml *Unwashed* sample was removed for testing (Fig 2.1). An equal volume of freshly prepared sterile isotonic diluent (5% dextran, 2% HSA in saline) was slowly added to the cryobag, with constant mixing, and the diluted cells transferred into a 600ml Baxter Fenwal 4R 2024 bag suitable for centrifuging, at 1400g for 10 minutes to pellet the cells. The speed of centrifugation was increased from the published 400g [Rubinstein et al., 1995], in order to improve separation of the cellular pellet and supernatant and reduce cellular loss during plasma expression. The supernatant, consisting of the DMSO cryopreservative and

Figure 2.1 Experimental Design



toxic cell lysis products, was removed by manual plasma expression and the cells resuspended in fresh dextran/saline to the original volume. Lastly, a 10ml *Washed* sample was removed for testing. The details of all reagents, disposables and equipment used in this laboratory validation are listed in Tables 2.1, 2.2 and 2.3 respectively.

Experimental Methods

Due to the logistics of simultaneously performing all assays at all time points, not all assays were performed on all bags. Sufficient repeats of each assay were performed in order to power the comparisons to the satisfaction of the statistician (Dr Wotherspoon).

Using time course the following parameters were investigated in the *Unwashed* and *Washed* samples at thirty minute time intervals for a period of ninety minutes; cell loss, overall viability, viable $CD34^+$stem cell enumeration, clonogenic potential and reduction in free haemoglobin content.

Cell counts and viability

At each time interval, a manual total cell count and cell viability was performed on a 1:100 dilution of samples and isotonic diluent solution. A manual cell count was then performed using a haemocytometer on a 1:1 dilution of cells to Trypan Blue solution.

Viable $CD34^+$stem cell enumeration

Using the total cell count obtained above, an appropriate sample dilution was prepared to obtain a volume of less than $100\mu L$ containing 8×10^5 nucleated cells. This was added to an Eppendorf tube containing fluorescently labelled monoclonal antibodies ($10\mu L$ each of CD45-FITC and CD34-PE), and $10\mu L$ of 7-AAD. A negative control tube was prepared identically except the anti-CD34 was substituted with an isotype control, IgG1-PE. Samples were incubated at room temperature in the dark for 15 minutes prior to addition of 100ul Optilyse B. After an additional 10 minutes incubation, $1ml$ distilled water was added. The samples were then incubated for another 10 minutes before immediate flow cytometric analysis.

A known concentration of ProCOUNT (Becton Dickinson) fluorospheres was added to each tube immediately prior to the addition of sample. This established a ratio of fluorospheres to the original volume of the sample, thus enabling the absolute $CD34^+$cell count to be calculated while eliminating the need for a WCC from a automated haematology analyser hence improving test reproducibility as well as the need to include

sample volume [Keeney et al., 1998, Chang and Ma, 1998, Rock et al., 2000, Dickinson and Company, 2003]. The incorporation of viability dyes such as 7AAD into single platform analysis allowed the determination of the absolute viable $CD34^+$cell count [Keeney et al., 1998]. Well-mixed samples were analysed using a Becton Dickinson FACScan flow cytometer with the CellQuest software package.

Through a series of sequential gates viable $CD34^+$stem cells are distinguished from all other cellular events by firstly identifying leucocytes, secondly viable leucocytes and finally viable $CD34^+$stem cells. The gating strategy is listed here in progressive order:

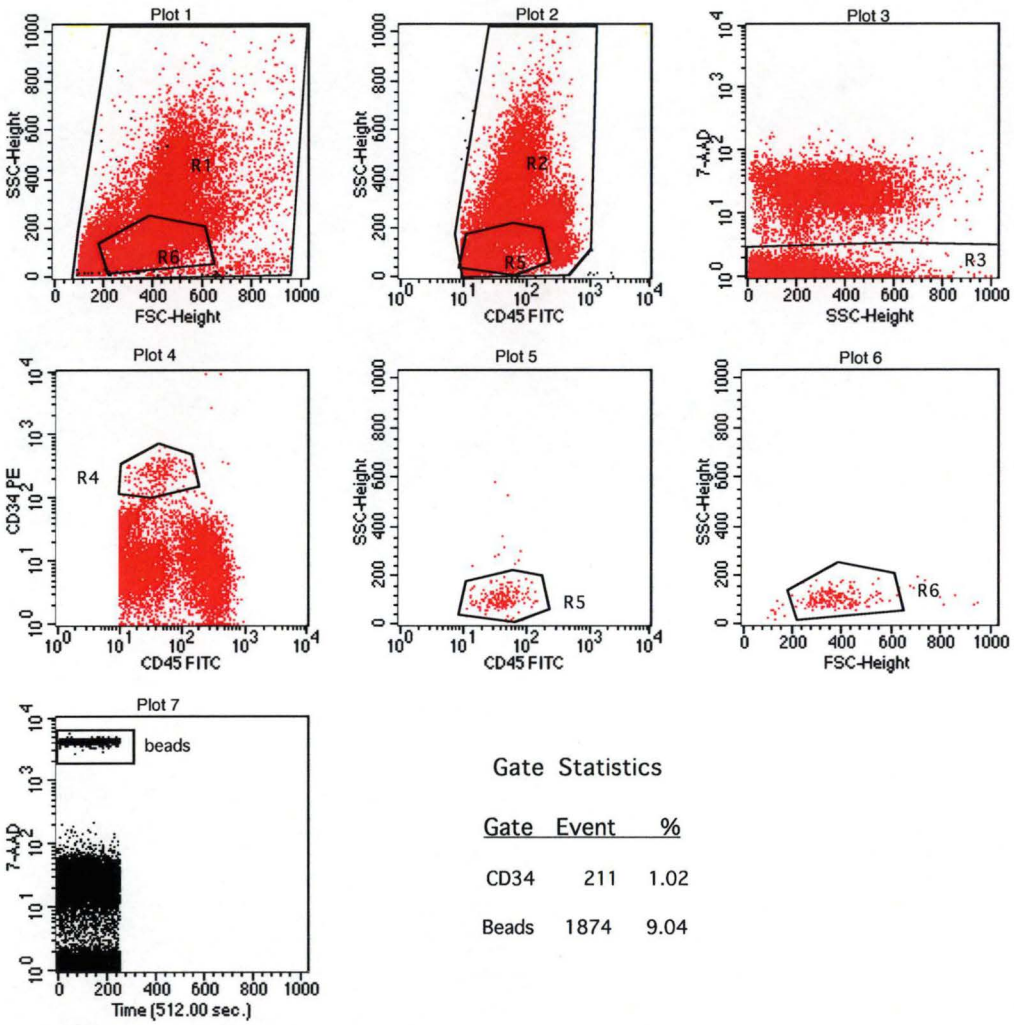
1. Viable $CD34^+$events present in *Washed* and *Unwashed* samples were isolated from other cellular and non-specific stained events because of a series of progressive logical gates (Fig. 2.2). The first histogram plot (plot 1) displays forward scatter vs side scatter for all events in which region 1 (R1) was set to include all cellular events. Plot 2 displays $CD45^+$events versus side scatter, with R2 set to collect all $CD45^+$events (i.e. leucocytes).

2. The first logical gate (G1) was set to include all of R1 and R2 and plot 3 was drawn using this gate. R3 was set to exclude dead cells, which stained with 7-AAD. These three regions were set by initially running samples in set-up mode prior to acquisition.

3. Viable leucocytes gated from R1, R2 and R3 are displayed in plot 4, which displays $CD45^+$vs $CD34^+$events, and R4 was set to select $CD34^+$SSC low events. Plot 5 displays CD45 versus SSC characteristics of $CD34^+$cells gated from additive regions 1-4. Any remaining non-specifically stained events such as debris, platelets and mature cells are excluded in R5 and so this region was set to select cells forming a cluster with the characteristic low SSC and low-intermediate CD45 fluorescence of HSC. The light scatter characteristics of the cells from additive regions 1-5 were displayed in plot 6, and R6 was set in the lymphoblastic region in order to isolate the cluster of events which fulfilled all the fluorescence and light scatter criteria of the $CD34^+$HSC. The number of events in R6 was used for the calculation of the absolute $CD34^+$cell count.

4. Single-bead events were collected into R7, which was set to only display the fluorospheres vs time, as prescribed by the manufacturer. The control sample was analysed using the same gating strategy. The calculation for absolute $CD34^+$was performed as per the instructions of the manufacturer [Dickinson and Company, 2003].

Figure 2.2



Gate Statistics

Gate	Event	%
CD34	211	1.02
Beads	1874	9.04

Bead Concentration = 1094 Dilution Factor = Neat CD34+/ul = 211/1874 x 1094 x 1 = 123.18/uL

Figure 2.2:Representative Example of Flow Cytometry Gating Strategy and Analysis for CD34+ events

5. The absolute $CD34^+$cell count per μL for each sample was calculated as follows:

$$\frac{CD34^+}{\mu L} = \frac{\#CD34^+\text{events}(R5)}{\#\text{beads counted}(R7)} \times \text{bead concentration} \times \text{dilution factor}$$

The same calculation was also performed on the control sample to determine the level of non-specific background staining. The $CD34^+$cell count/μL was calculated from the test samples, and the absolute $CD34^+$ cell count was determined by subtracting the control sample result from this value.

CFU-GM Clonogenic Assays

CFU-GM assays were performed on the *Washed* and *Unwashed* samples to assess the clonogenic potential. CFU-GM assays were set up in duplicate for both *Washed* and *Unwashed* samples at thirty-minute intervals over a ninety-minute period post-thawing.

Using the total viable cell count obtained from the manual cell count, an appropriate sample dilution was prepared to obtain aliquots containing 20 000 or 50 000 viable nucleated cells (depending on cell numbers) in $300\mu L$ volume. This was added to 1 ml of Methocult GFH4534 (Stem Cell Technologies), a commercially available, semi-solid medium containing recombinant cytokines, and 1.1 ml plated onto duplicate gridded 35mm diameter petri dishes and incubated for 14 days at 37 °C in a humidified atmosphere containing 5% CO_2. Colonies were defined as clusters of 30 cells and were identified using an inverted microscope. The results obtained were used to calculate the mean number of CFU-GM per 10^4 viable nucleated cells.

The total number of CFU-GM in the harvest was calculated as follows:

$$\text{Total CFU-GM} = \frac{\text{Mean CFU-GM}}{10^4\ \text{VNC}} \times \frac{\%\ \text{viability}}{100} \times \frac{\text{TNC}}{10^4}$$

where VNC is the viable nucleated cells and TNC is the total nucleated cells of the final product.

Free Haemoglobin Content

Assessments of free haemoglobin levels were performed on cell-free supernatants of *Washed* and *Unwashed* samples following centrifugation at 7000g for thirty seconds in a microfuge. Dipstick analysis (Hemastix, Bayer) was performed on 1:25000 dilution (isotonic diluent solution) of the supernatant

Table 2.4: Free Haemoglobin

Free Haemoglobin (Red Cell Equivalent/uL)	Score
< 25	1
$25 - 80$	2
80	3
$80 - 200$	4
> 200	5

and the results (in red cell equivalent/ul) read after 60 seconds. Categories were assigned to diluted haemoglobin levels, as indicated in Table 2.4, to allow Sign test statistical analysis.

Statistical Analysis

Ms Imogen Liew, University of Tasmania provided statistical advice and guidance. Univariate and Student's paired t-test statistical analysis was performed on sample data with the criterion for significance determined as a p value of $P < 0.05$.

2.3 Results

2.3.1 Loss of Cells During Washing Procedure

The rate of centrifugation was increased from 400 g to 1400g in order reduce the potential for cell losses by improving the separation between the cell pellet and supernatant. Manual cell counts (using Trypan Blue solution) were obtained immediately post-manipulation in order to assess the effect of the washing protocol on cell numbers for *Unwashed* and *Washed* samples (Table 2.5), where PBSC is Peripheral Blood Stem Cell Collection and BMH is Bone Marrow Harvest.

Student's paired t-test analysis of the data demonstrated no significant difference cell counts between *Washed* and *Unwashed* cells ($p = 0.10$) as displayed in Table 2.5. Further analysis found no significant difference existed between samples obtained from BMH ($p = 0.30$) or PBSC ($p = 0.30$) harvests. The average recovery of cells of $95 + 8.0\%$ confirmed these findings. Therefore, the washing protocol did not result in significant loss of cell numbers whilst centrifugation at 1400g improved the separation of the supernatant and cell pellet.

Table 2.5: Comparison of Total Nucleated Cells ($\times 10^8$) for samples post-thawing.

PBSC		BMH	
Unwashed	*Washed*	*Unwashed*	*Washed*
343.2	351.0	126.9	112.5
284.7	240.5	107.7	115.7
325.5	299.5	60.5	59.4
317.2	322.4	345.6	292.8
66.6	60.7		
309.4	319.8		
$p = 0.30$		$p = 0.30$	
$p = 0.1$			

As there was no difference between PBSC and BMH these values were combined in subsequent analysis except where noted.

Viability of Cells After Washing

The changes in total cell viability percentage values using time course (commencing immediately post-thawing) for both *Washed* and *Unwashed* samples are shown in Table 2.6.

The viability of *Washed* and *Unwashed* cells, compared by Student's paired t-test analysis on samples at thirty-minute intervals over a ninety-minute period, shows that at each time point, viability is improved for *Washed* samples. The average of each time point shown in Figure 2.3 shows strong evidence of improved viability of *Washed* cells in comparison to *Unwashed* cells over time ($p = 0.018$). Under univariate analysis, the rate of loss of viability is also significantly less in *Washed* cells compared to in *Unwashed* cells ($p = 0.005$) indicating that *Washed* cells are dying at a slower rate than their *Unwashed* counterparts.

Although not measured in a quantitative manner, the incidence of cell clumping was visually assessed to be reduced in the *Washed* samples in comparison to the *Unwashed* samples. Although this is at best a qualitative observation, decreased clumping of *Washed* cells does correlate with their increased viability.

In conclusion, cryopreserved stem cells that were *Washed* with an isotonic diluent solution exhibited improved viability and a slower rate of subsequent death compared to *Unwashed* controls.

Figure 2.3

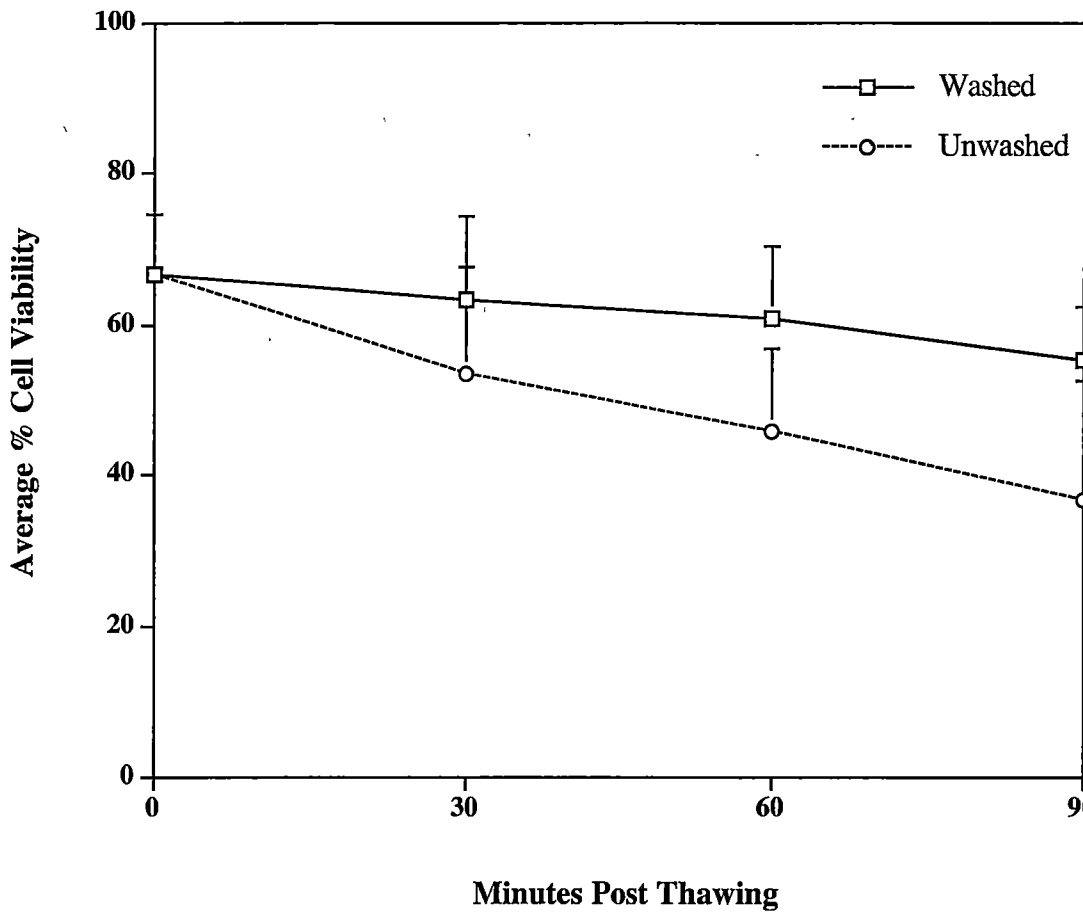


Figure 2.3:Comparison of Nucleated Cell Viability of Washed and Unwashed Products

Thawed cells were either Washed as per Methods (solid line) or left Unwashed (dashed line) and stained with Trypan Blue. Viable and non-viable cells were enumerated using a haemocytometer. Results shown are Mean ± SD of 10 experiments. There is a significant increase ($p = 0.005$) in nucleated cell viability in Washed samples in comparison to Unwashed samples.

Table 2.6: Comparison of the percentage viability for *Washed* and *Unwashed* samples at each time point.

Immediately	30 mins		60 mins		90 mins	
post-thaw	*Unwashed*	*Washed*	*Unwashed*	*Washed*	*Unwashed*	*Washed*
65.9	41.6	68.0	43.4	41.6	25.8	55.0
63.7	43.8	47.9	38.6	43.8	26.6	49.6
61.0	49.0	49.0	36.0	49.0	22.0	58.0
71.0	42.0	52.0	32.0	42.0	23.0	49.0
63.0	44.8	56.0	44.9	44.8	34.6	46.6
82.6	82.0	87.2	63.5	82.0	61.8	69.2
69.1	64.0	64.0	52.0	64.0	42.3	55.9
56.1	59.8	57.1	57.6	59.8	58.0	58.7
55.0	41.0	43.0	25.0	41.0	22.0	51.0
73.0	36.0	69.0	26.0	69.0	29.0	69.0
p value	< 0.05		< 0.001		< 0.001	

2.3.2 Enumeration of Viable $CD34^+$Haematopoietic Stem Cell

The next step was to establish if the improved cellular viability extended to HSCs by enumeration of viable $CD34^+$stem cells. Using single platform flow cytometry, the number of viable $CD34^+$stem cells was determined in *Washed* and *Unwashed* samples. Testing was performed at thirty-minute intervals post-thawing over a ninety-minute period (Table 2.7). The numbers shown in Table 2.7 represent the total number of $CD34^+$HSC (x 10^6) present in the entire *Washed* and *Unwashed* harvests. The effect of washing on the number of viable $CD34^+$HSC viability was assessed by paired t-test analysis and multivariate analysis of variance.

Student's paired t-test analysis of the absolute $CD34^+$numbers at each time point shows that, although there was no significant difference between *Washed* and *Unwashed* samples (Table 2.7), the differences at latter time points suggests a trend towards enhanced CD34 viability in *Washed* samples. However, further analysis of the data using multi-variate analysis shows that the difference in absolute numbers of $CD34^+$stem cells in the *Washed* and *Unwashed* cells was not statistically significant ($p = 0.998$), indicating that washing did not reduce the number of viable $CD34^+$cells relative to *Unwashed* controls.

Figure 2.4 represents the average percentage of original viable $CD34^+$cells

Figure 2.4

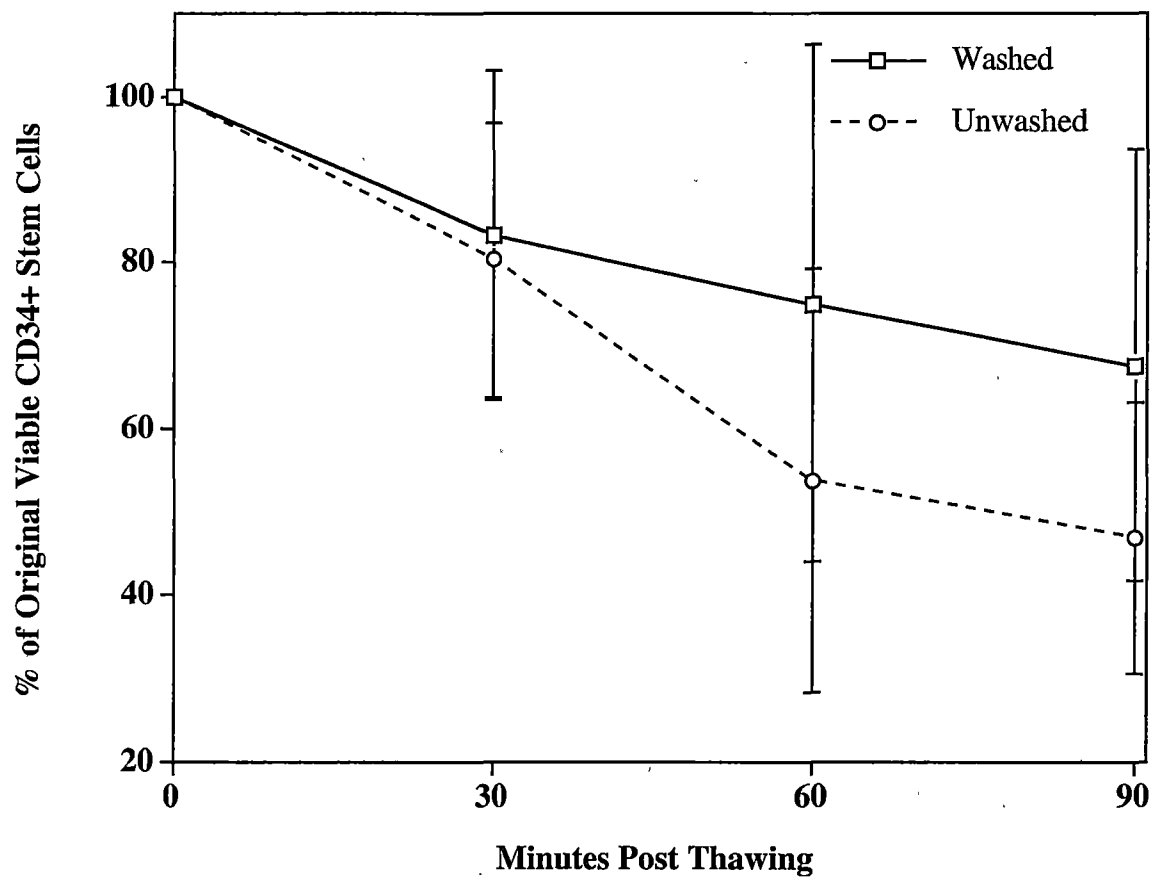


Figure 2.4:Percentage Changes in Viable CD34$^+$ Stem Cell Content Over Time.

Thawed cells were either washed as per Methods (solid line) or left Unwashed (dashed line) and viable CD34$^+$ HSC enumerated using single platform flow cytometry. The changes in the number of viable CD34$^+$ HSC are expressed as a percentage of the original count immediately post-thawing. Results shown are Mean ± SD of 7 experiments. Using univariate analysis there is no evidence of a difference in CD34 counts between treatments ($p = 0.998$).

over time. For graphical purposes, the immediate post-thaw samples were enumerated for $CD34^+$cells and this value was designated as 100% and the subsequent 30, 60, 90 minutes $CD34^+$cell counts are expressed as a percentage of the immediate post-thaw $CD34^+$HSC count. Figure 2.4 demonstrates that the difference in the average percentage of original $CD34^+$counts for the *Washed* and *Unwashed* cells over time was minimal for the early time points, although there does appear to be a trend towards improved viability for *Washed* $CD34^+$cells with time. However, multi-variate analysis shows evidence of a constant rate of decrease in the $CD34^+$count over time ($p = 0.002$) indicating that stem cells continue to lose viability post-thawing.

In conclusion, the washing protocol does not diminish viable $CD34^+$HSC numbers in comparison to *Unwashed* cells, indicating that HSC viability is not adversely affected by the washing of thawed harvests. Furthermore, the trend towards significance in absolute viable $CD34^+$HSC counts over time between *Washed* and *Unwashed* samples, suggests washing may improve the viability of $CD34^+$HSC.

2.3.3 CFU-GM Assays

Although viable $CD34^+$HSC numbers were not adversely affected by the washing of thawed HSC harvests, it was also necessary to assess the impact of the washing protocol on the clonogenic potential of HSC. This was assessed by CFU-GM assays that were performed at thirty-minute intervals post-thawing over a ninety-minute period (Table 2.8) and the data analysed by Student's paired t-test analysis and multivariate analysis of variance. The numbers shown in Table 2.8 represent the total number of CFU-GM present in the entire *Washed* and *Unwashed* harvests tested.

Student's paired t-test analysis of absolute CFU-GM assay values at each time point, suggests that although there is no significant difference at any of the time points ($p > 0.05$), there is a trend towards significance from the sixty-minute time point onwards (Table 2.8). Multivariate analysis confirms that the difference in CFU-GM formation between *Washed* and *Unwashed* cells over time is not statistically significant ($p = 0.686$).

Figure 2.5 displays the average relative percentage of CFU-GM values over time in relation to the immediate post-thaw CFU-GM value that was designated as 100%. This figure illustrates that the difference in counts for the *Washed* and *Unwashed* cells over time was minimal for the early time points. However, *Washed* CFU-GM values do appear to improve with time (relative to *Unwashed* cells) as noted with the statistical analysis of the absolute numbers.

In summary, the washing protocol does not have a detrimental affect on

Figure 2.5

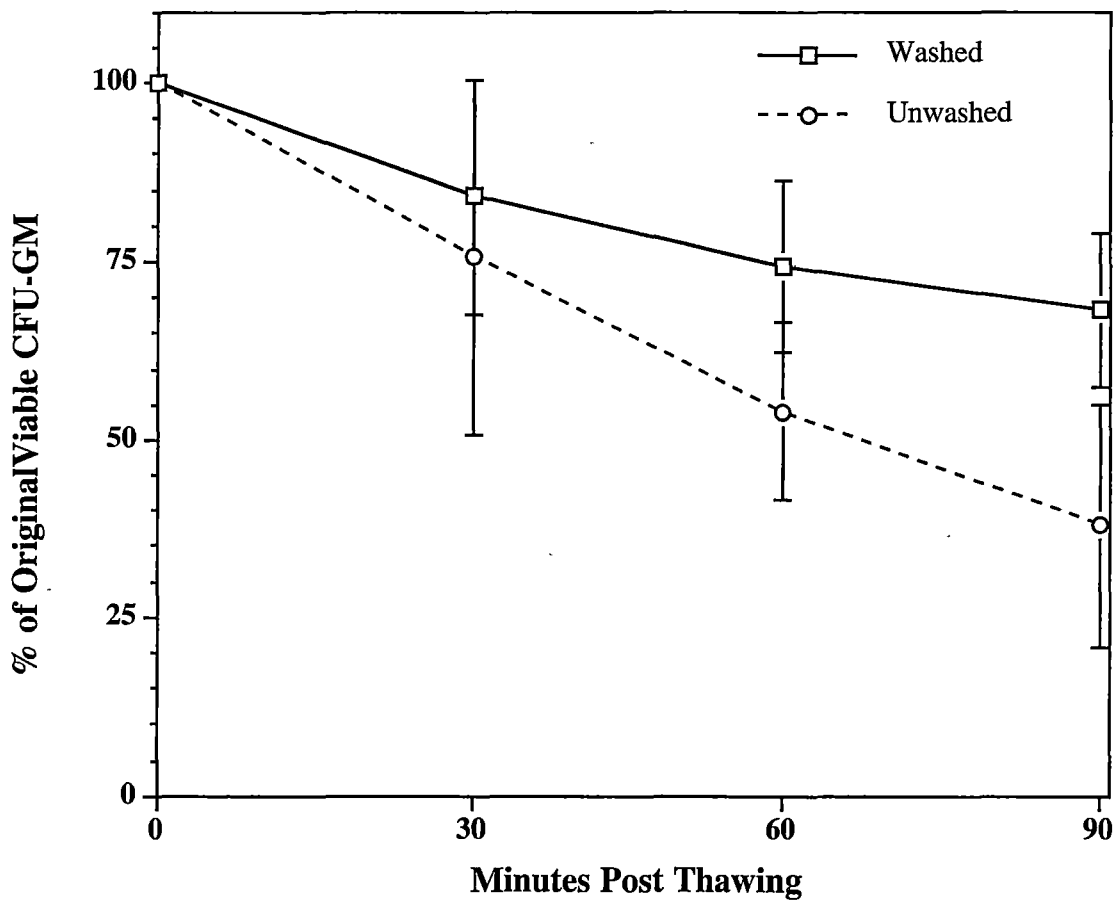


Figure 2.5: Percentage Changes in CFU-GM Formation Over Time

Thawed cells were either washed as per Methods (solid line) or left Unwashed (dashed line) and placed into CFU-GM cultures, which were enumerated after 14 days incubation. The changes in the number of CFU-GM recovered are expressed as a percentage of the sample taken immediately post-thawing. Results shown are Mean ± SD of 5 experiments. The observed difference in CFU-GM formation between washed and unwashed cells was not statistically significant ($p = 0.686$).

Table 2.7: Comparison of total viable numbers of $CD34^{+}(\times 10^{6})$ HSC for entire *Washed* and *Unwashed* harvests at each time point.

Immediately	30 mins		60 mins		90 mins	
post-thaw	*Unwashed*	*Washed*	*Unwashed*	*Washed*	*Unwashed*	*Washed*
409.3	295.4	323.1	253.4	312.7	216.0	301.1
26.37	20.7	25.6	.....	24.3	12.4	24.8
66.7	65.5	67.6	57.7	70.9	33.5	58.1
60.5	47.9	49.8	27.9	30.6	13.4	28.1
103.8	51.5	67.4	34.3	56.2	50.8	49.7
302.8	274.3	319.0	340.5	359.2	224.7	285.4
75.5	70.9	38.9	26.9	33.7	24.8	22.9
p value	0.30		0.06		0.07	

Table 2.8: Comparison of total numbers of CFU-GM $(\times 10^{5})$ for entire *Washed* and *Unwashed* harvests at each time point

Immediately	30 mins		60 mins		90 mins	
post-thaw	*Unwashed*	*Washed*	*Unwashed*	*Washed*	*Unwashed*	*Washed*
62.5	47.0	41.0	31.0	42.0	31.0	43.0
56.0	19.9	51.5	19.9	45.1	7.7	44.3
221.1	173.3	233.4	140.2	204.4	116.0	169.0
97.7	101.2	85.4	65.8	65.0	46.5	43.9
167.9	144.5	117.3	92.0	114.4	44.1	107.1
p value	0.63		0.09		0.06	

the potential of committed myeloid markers (a surrogate indicator of HSC functionality) to proliferate relative to *Unwashed* controls. Moreover, the results indicate that washing may even improve the ability of the HSCs to engraft and mature into macrophages and granulocytes, in comparison to *Unwashed* controls after sixty minutes post-thawing, as indicated by the trend towards significance.

2.3.4 Reduction in Free Haemoglobin

The effectiveness of the washing protocol to remove free haemoglobin from bone marrow harvest and peripheral blood stem cell collections was assessed by comparing the amount of haemoglobin present in the supernatant of *Washed* and *Unwashed* samples. Supernatants were diluted 1/25000 prior to analysis. The results were separated into bone marrow harvest and peripheral blood stem cell collections as the red cell concentration of bone marrow harvests was usually greater.

Free haemoglobin levels in both *Washed* bone marrow and peripheral blood stem cell harvests were significantly reduced in comparison to *Unwashed* supernatants ($p =< 0.05$, Sign Test). As can be seen in Figure 2.6, *Unwashed* bone marrow harvests contained greater amounts of free haemoglobin than the *Unwashed* peripheral blood stem cell harvests and washing achieved significant reductions in the haemoglobin content of both stem cell sources. On average, a 4−fold reduction in free haemoglobin was achieved for bone marrow harvests and 3.6-fold reduction for peripheral blood stem cell collections. However as several bone marrow samples were recorded as > 200 RCE/ul (the upper limit of the dipstick test) and would have required further dilution, the degree of reduction for bone marrow harvests was most likely underestimated. However, as the levels of free haemoglobin were clearly reduced in *Washed* samples further dilution was not deemed necessary.

Figure 2.7 compares the free haemoglobin scores of the *Unwashed* and *Washed* supernatants for each individual sample illustrating the general trend in free haemoglobin reduction after washing.

In summary, washing of stem cell products achieved a significant reduction in the amount of free haemoglobin present in the supernatants of both thawed bone marrow and peripheral blood harvests.

Figure 2.6

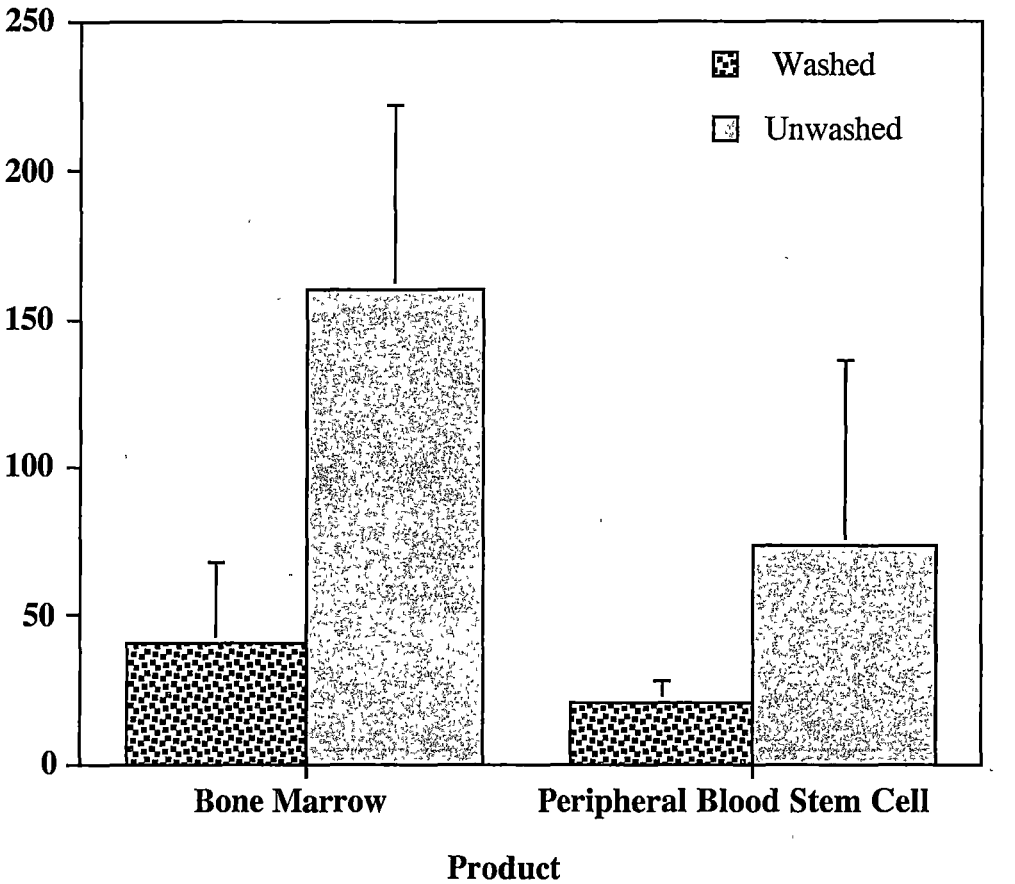
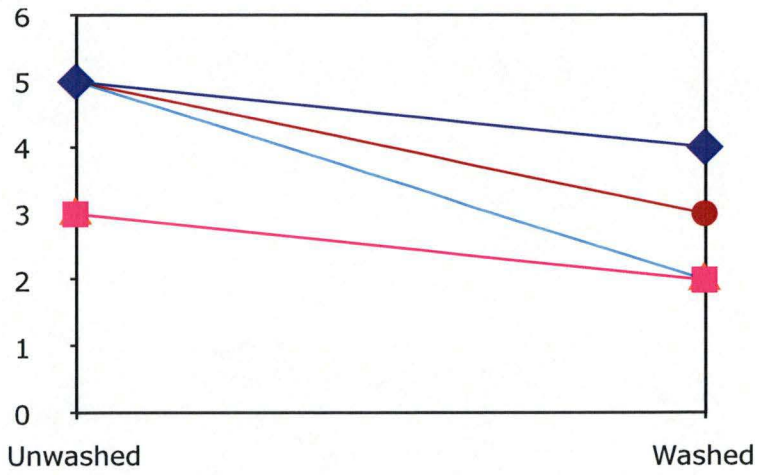


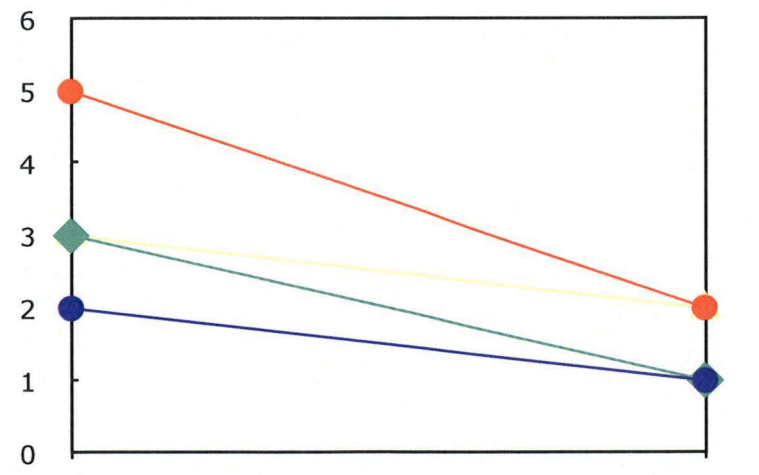
Figure 2.6: Free Haemoglobin Content of Washed and Unwashed Bone Marrow and PBSC Harvests.

Cell-free supernatants were created from samples taken immediately post-thawing (unwashed, solid column) and post-washing (washed, large dotted column) and diluted 1/25000. Free haemoglobin (in Red Cell Equivalent /ul) was measured in the diluted specimens using a dipstick test. Results shown are Mean ± SD of 7 experiments for both bone marrow and PBSC harvests.

Figure 2.7



Bone Marrow Harvests



PBSC Harvests

Figure 2.7: Free Haemoglobin Score of Washed and Unwashed Bone Marrow and PBSC Harvests.

Cell-free supernatants were created from samples taken immediately post-thawing (unwashed, solid column) and post-washing (washed, large dotted column) and diluted 1/25000. Free haemoglobin (in Red Cell Equivalent /ul) was measured in the diluted specimens using a dipstick test and was assigned a Free Haemoglobulin Score. Results shown are of 7 experiments for both bone marrow and PBSC harvests.

2.4 Discussion

Autologous HSC transplantation involves the intravenous infusion of HSC collected from the patient's own bone marrow or peripheral blood to replenish haematopoietic cell function after high dose chemotherapy [Armitage, 1994]. DMSO, a cryoprotectant, is added to cells prior to cryopreservation and subsequent storage in liquid nitrogen to protect the cell membrane integrity by preventing the formation of ice crystals [Rowley, 1992]. Consequently, when the patient is reinfused they receive DMSO along with ruptured granulocytes and red cell debris (DMSO poorly preserves these cells). The infusion of cryopreserved cells has been associated with a number of DMSO and cellular lysis related toxicities including nausea, vomiting, acute renal failure, and cardiac and pulmonary arrest [Davis et al., 1990]. The washing of HSC post-thawing may reduce these side effects through the removal of DMSO and cellular lysis products. Nonetheless, before such a procedure can be implemented, validation studies are mandatory.

Validation studies are crucial because any amendments to a clinical practice require elucidation of all consequences of the modifications. Regulatory bodies such as the National Pathology Accreditation Advisory Council (NPAAC) and the National Association of Testing Authorities (NATA) also require documentation of the validation procedures for any changes, prior to implementation as part of their accreditation process. Approval from these bodies is required for laboratory operation.

This chapter reports on a validation study of a washing protocol in which the rate of centrifugation was increased to 1400g to improve the separation of the HSC pellets from the supernatant in order to reduce cell loss during plasma expression. The NYCBB washing protocol described by Rubinstein et al. 1995 concluded that DMSO could be removed from thawed cryopreserved cord blood by washing without affecting HSC cell viability or clonogenic ability following centrifugation at 400g. However cell loss remained a concern with this protocol with one study reporting a median loss of 27.2% (range 11.1 to 41.2%) of total nuclear cells in a study of 12 cord bloods [Antonenas et al., 2001]. The increased centrifuge speed used in this investigation resulted in improved sedimentation of cells, increased density of the cell pellets, and hence overall improved separation of cell pellets from supernatant. As desired, the removal of the supernatant (containing DMSO, red cell debris and ruptured granulocytes) by plasma expression was achieved without disturbing the cell pellets.

The increase in centrifugation rate however raised the spectre of damage to HSC, therefore, multi-parameter testing was used to determine the impact of the revised washing protocol. Cell recoveries and viabili-

ties [Hartmann et al., 1985] were assessed by manual cells counts, while the impact on engraftment potential [Spitzer et al., 1980, Audet et al., 1998] was measured by viable $CD34^{+}$HSC enumeration and CFU-GM assays of *Washed* and *Unwashed* samples. The effectiveness of the washing protocol to remove contaminants such as DMSO, red cell debris and ruptured granulocytes was assessed by the measurement of free haemoglobin in *Washed* and *Unwashed* samples.

The effect of the modified washing protocol on total cell numbers was assessed by manual cell counts on *Washed* and *Unwashed* samples. The findings of this study compare with the findings of Rubinstein et al.1995 which reported no significant difference in nucleated cell count after washing using the NYCBB washing protocol. Previous studies [Ragab et al., 1977, Broxmeyer et al., 1989] have expressed concern that the removal of DMSO by washing may reduce the number of HSCs available for reinfusion. This study allays these fears by demonstrating that there is no significant loss of cell numbers using the revised washing protocol. Moreover, the observation that washing preserves the viability of cells over a ninety-minute interval compared to *Unwashed* cells extends the findings of Rubinstein etal 1995 who used a single time-point. Thus, this study demonstrates that the removal of DMSO, lysed red cells and ruptured granulocytes, in conjunction with the re-suspension of stem cells in isotonic diluent solution significantly improves the viability of stem cells post-thawing.

As the measurement of cell clumping by quantitative methods was not possible, the impact of the washing protocol on cell clumping could only be assessed qualitatively. The reduction in cell clumping in *Washed* cells observed in comparison to *Unwashed* cells in this study suggested the ruptured granulocytes are removed by the washing process. Cell losses of up to 50% have been reported in the presence of damaged granulocytes [Rowley, 1992] demonstrating that clumping has the potential to detrimentally reduce the dosage of HSCs reinfused to patients. Furthermore, the release of contents of the ruptured granulocytes (such as histamine) has been linked to reinfusion related toxicities [Okamoto et al., 1993]. The washing protocol has the potential to reduce the loss of HSCs through cell clumping as well as toxicities related to the reinfusion of ruptured granulocytes and so improves on current practice.

The enumeration of $CD34^{+}$HSC is routinely used to assess the engraftment potential of stem cell collections [Keeney et al., 1998]. The finding of no significant difference in viable $CD34^{+}$HSC counts between *Washed* and *Unwashed* cells demonstrates that the increased centrifugal speed does not adversely affect viable HSC numbers. The average percentage change over time in the post-thaw $CD34^{+}$HSC count was used for graphical presentation

in this study as the $CD34^+$HSC content of the individual harvests tested varied immensely (from adequate HSC numbers for engraftment to grossly insufficient).

The trend towards significantly improved viability of the *Washed* samples from the sixty minute time points suggests that washing may in fact improve the viable $CD34^+$numbers relative to *Unwashed* cells. These findings provide further demonstration of the safety of the modified washing protocol as well as the possibility that it may improve the viability of HSC post-thawing. Whilst testing of additional harvests may demonstrate a significant difference over time for *Washed* samples, the purpose of this study was to demonstrate that the washing protocol did not adversely affect HSC. Ultimately this study therefore represents an improvement upon the original study [Rubinstein et al., 1995], which did not perform viable $CD34^+$HSC analysis.

Colony Forming Units Granulocyte Macrophage (CFU-GM) assays are routinely performed as an in-vitro measure of the haematopoietic progenitor content of a stem cell collection [Eaves and Lambie, 1995]. In this study, the difference in CFU-GM formation between *Washed* and *Unwashed* cells was not statistically significant. The absolute values of CFU-GM assays of the harvests used in this study were also reported as the average percentage change in the original post-thaw value over time for graphical representation because of the dramatic variation between the individual harvests tested.

The trend towards significance of the *Washed* samples from the sixty minute time points suggests that washing may in fact improve the CFU-GM assays of *Washed* relative to *Unwashed* cells. The performance of additional testing may confirm a significant improvement in clonogenic potential of *Washed* over time, however as stated earlier, this was not the aim of the study. When taken together with the results of the Rubinstein etal 1995 study, which reported that the clonogenic activity of HSC post washing was improved by the removal of DMSO, the data demonstrates that this procedure will not adversely affect clonogenic potential.

To avoid DMSO-related post-thaw toxicity to HSC [Bostrom and Burger, 1999], conventional HSC reinfusions occur within minutes of thawing. This study shows significant improvement of overall cell viability as well as a trend towards improving viable $CD34^+$and CFU-GM recovery over time indicating that washing reduces the rate of cell loss and cell death. The modified washing protocol should therefore be considered as a cell-saving strategy in the event of an unexpected delay (e.g loss of central line) of reinfusion of thawed products.

The dramatic reduction of free haemoglobin levels in *Washed* samples is a consequence of the removal of red blood cell debris from the

cryopreserved stem cell product. As this study was unable to measure DMSO directly by gas chromatography, free haemoglobin was used as a surrogate marker of dilutional effects. Therefore by extrapolation of the free haemoglobin results, the DMSO concentration must also be accordingly reduced. Furthermore, the findings of this study support the estimation of Rowley et al 1999 that the NYCBB washing protocol removed 90% of DMSO from cryopreserved stem cells. Kessinger et al., 1990 found that of 100 patients, 92% experienced haemoglobinuria and 64% experienced vomiting post reinfusion of cryopreserved HSC. Several studies of autologous haematopoietic stem cell transplantation related toxicities [Davis et al., 1990, Stroncek et al., 1991] have suggested that decreasing the amount of DMSO will reduce the incidence of these and other side effects. The total DMSO dose reinfused into patients is generally restricted to no more than 1g DMSO/Kg/day [Bostrom and Burger, 1999]. The modified washing protocol is an effective method of reducing DMSO volume and hence enables increased reinfusion volume of cryopreserved cells to be tolerated.

2.5 Summary

This chapter has modified the NYCBB HSC washing protocol [Rubinstein et al., 1995] by improving the separation of cell pellet and supernatant and subsequently validated this change in methodology. In line with the original report, this study found improved cell viability and no loss of nucleated cells or CFU-GM numbers. Furthermore, there was no loss of $CD34^{+}$HSC and the reduction in free haemoglobin levels indicated the removal of cellular lysis products and, indirectly, DMSO. In addition, analysis of the effect of washing on these parameters over an extended time frame found that the modified washing protocol removed red cell lysates and DMSO without affecting the HSC numbers or engraftment potential. Therefore, the laboratory data indicates that this procedure would be suitable for use in the patient setting. This protocol could also be used in the laboratory in the event of delays in reinfusing HSC post-thawing in order to improve cell viability and to slow the gradual loss of HSC numbers and clonogenic potential over time.

The modified HSC washing protocol is a feasible practice that reduces the morbidity associated with the reinfusion of cryopreserved stem cells without adversely affecting HSCs. A clinical trial will be necessary prior to systemic adoption of the washing protocol to determine if the procedure is of significant benefit to patients.

Chapter 3

Clinical Trial

3.1 Introduction

The aim of this thesis was to investigate if washing of thawed stem cell products prior to reinfusion significantly reduces the morbidity associated with this procedure. This clinical trial used the modified version of NYCBB's washing protocol (Rubinstein et al. 1995) which had beenpreviously validated in a laboratory study. This was a non-randomised trial to assess the side effects experienced by patients undergoing ASCT at the Royal Hobart Hospital, as well as the reactions of both their carer and attending nurse, using VASQOL surveys.

We believe that washing of thawed HSC prior to reinfusion will reduce the side effects experienced by patients, carers and nurses. Therefore a formal clinical trial was required to properly study whether this proposed change in clinical practice was of actual value to those individuals involved. Hence, the need for a clinical trial of this procedure in the patient setting, studying the impact of the washing procedure on patients receiving ASCT, in comparison to current practice.

For the purpose of this thesis it was necessary to use a QOL survey to gain information regarding side effects associated with ASCT from the perspective of individuals involved. We decided to use a modified version of a VASQOL survey previously developed by Rowley 1999 to assess impact of post-thaw washing of symptoms associated with ASCT including nausea, pain, flushing, nervousness, shortness of breath, unusual smells and tastes.

Reagents	Suppliers
0.9 % Saline Infusion BP	Baxter
20% Human Serum Albumin (HSA)	CSL Ltd
Gentran 40 Dextran 40 Intravenous Infusion BP 10% w/v in NaCl	Baxter
Intravenous Infusion BP 0.9 w/v	

Table 3.1: Reagents and Suppliers

Disposables	Manufacturers
60 ml syringe with Luer lock	Terumo
18 G Drawing up needle, non-bevelled	Braun
1000ml Fenwal Transfer pack container with coupler	Baxter
600ml Fenwal Transfer pack container with coupler	Baxter
Sampling site coupler	Fenwal
Hemastix	Bayer
Reinfusion line IV set non filtered	Portland Surgical
Bactec Peds Plus/F aerobic	BD
Bactec Plus+ Anaerobic/F	

Table 3.2: Disposables and Manufacturers

Equipment	Manufacturers
400 Cyclic Fridge	Kelvinator
Class II Biological Safety Cabinet	Gelman Sciences Australia
PB 3000 Scales	Mettler
Plasma Extractor	Fenwal
RF Sealer Model 2101	Sebra
Varifuge 3.0RS	Heraeus Instruments
Tubing Welder	Terumo
Waterbath	Haake

Table 3.3: Equipment and Manufacturers

3.2 Materials and Methods

3.2.1 Maintenance of Sterility

Sterility was maintained with a Class II Biological Safety Cabinet where appropriate.

3.2.2 Solutions

Isotonic Diluent Solution

Gentran	500 ml
Saline	400 ml
HSA	100 ml

Components were mixed and stored at 4 °C.

3.2.3 Study Samples

Ethical Approval

Approval for this study was obtained from the Royal Hobart Hospital Human Research Ethics Committee (Project No. H6737).

Selection of the Study Samples

All patients undergoing ASCT at the Royal Hobart Hospital were eligible for this study and hence all were asked to undertake the VASQOL survey. Patients who had a second transplant dose remaining in storage with a minimum CD34 count of 2×10^6/kg were invited to participate in the Test Arm of the trial and hence receive *Washed* stem cells i.e. patients required 2 transplants, each with a CD34 count of at least of 2×10^6/kg, to be considered eligible for the Test Arm. Patients who declined to participate in the Test Arm received *Unwashed* cells (as per current practice).

Patients who had only a single transplant dose available were invited to undertake the VASQOL survey as a member of the Control Arm. These patients received *Unwashed* stem cells as per current practice. Additionally, patients who declined to participate in the Test Arm were invited to join the Control Arm.

Those patients who do not consent to completing the VASQOL survey were excluded from the trial as were patients who had a total transplant dose of less than 50mL (as these patients do not suffer sufficient side effects to warrant reduction by washing). These patients received *Unwashed* stem cells as per usual practice.

Sample Size

The main concern with QOL studies is whether or not there is sufficient power in the trial to answer the hypothesis. An Associate Investigator on this project, Dr. Simon Wotherspoon, performed power analysis to determine approximately how many subjects are needed in each arm to obtain measurable differences between the two groups. Power analysis using repeated measurements requires care as the power of the test is dependent not only upon the variability of observations, but also the level of the correlation amongst the repeated measurements. In order to obtain sufficient power for statistical analysis, it was recommended to employ 20 subjects per arm.

3.2.4 Experimental Design

During the four year course of the clinical trial 43 patients enrolled in the *Unwashed* control arm and 19 patients enrolled in the *Washed* test arm. On the day of reinfusion, prior to the administration of pre-medication, nursing staff recorded baseline clinical observations on the *reinfusion nursing documentation record* (Figure 3.1), including the free haemoglobin level of urine. After the administration of pre-medication, the nursing staff repeated all clinical observations and documented them on the reinfusion nursing record. At this time the patients, their carers and nurses were asked to complete their Pre-Reinfusion VASQOL surveys.

Using the post thaw washing method (previously validated in the laboratory phase of this study), the frozen cryobags containing stem cell collections of patients enrolled in the *Washed* test arm were rapidly thawed in a waterbath at 37 °C. Using aseptic techniques, the bags were spiked with a blood pack adaptor and an equal volume of freshly prepared sterile isotonic diluent (5% dextran, 2.0% HSA in saline) was slowly added to the cryobag, with constant mixing, and the diluted cells transferred into a 600ml Baxter Fenwal 4R 2024 bag suitable for centrifuging, at 1400g for 10 minutes to pellet the cells. The supernatant, consisting of the DMSO cryopreservative and toxic cell lysis products, was removed by manual plasma expression and the cells resuspended in fresh dextran/saline to the original volume. The *Washed* HSCs were then transported to the patient bedside and immediately reinfused into the patient approximately 35 minutes after thawing had commenced.

In the event that multiple cryobags were requested for reinfusion, a maximum of two bags would be thawed simultaneously in the laboratory by two scientists, diluted with isotonic diluent solution and then combined into one bag for centrifugation and further processing. The thawing of additional bags would only commence once the reinfusion of previous washed HSC had been

Figure 3.1

REINFUSION NURSING DOCUMENTATION RECORD

Patient Name: _____

Patient: Ur.No: _____

Time of Reinfusion: _____

Date of Reinfusion: _____

Volume Reinfused: _____mls

0= None	1=Mild	2=Moderate	3=Severe	4=Life Threatening

NOTE: Please complete all items for the pre-infusion symptoms. You do not need to record symptoms that do not occur subsequently. Please attach additional sheets as needed.

Event	Time	T	P	R	BP	Pain/ Location	CNS	Cough	Rash/ Flushing	Chills	Nausea	Vomiting	Diarrhoea	Urine Hemastix Test
Baseline														
Post Pre-Med														
End of Reinfusion														

successfully completed.

The frozen cryobags of the patients enrolled in the *Unwashed* arm of the trial were thawed at the bedside in a 37C waterbath immediately prior to reinfusion.

On completion of the reinfusion patients, their carers and nurses were asked to complete the Post-Reinfusion VASQOL survey. Nursing staff repeated all clinical observations at regular intervals until 6 hours post reinfusion (if reinfusion occured as an in-patient procedure) or at time of discharge (if reinfusion was an out-patient procedure). Twenty-four hours post reinfusion the patients, their carers and nurses were asked to complete the final VASQOL survey. All data collected from VASQOL surveys from the test and control arm was assigned a non-identifying collection number, which was used for all analysis.

Microbiological testing was also performed on the expressed supernatant of all *Washed* HSCs to assess the potential for contamination as a result of the post-thaw washing.

VASQOL survey

The toxicity of the ASCT was measured using a 10cm straight line where each end represents the extreme limits of the symptom ie 'no nausea' on one end to 'worst possible nausea' on the opposite end (Figure 3.2). The subject was asked to place a mark along the line to indicate their experience of the symptom [Gaston-Johansson et al., 1992]. We also extended the subject group in comparison to [Rowley et al., 1999a], to include side effects experienced by family members ("carers") (Figure 3.3) and nursing staff (Figure 3.4) as a consequence of contact with DMS exhaled by the patient as they metabolised DMSO. Furthermore, in contrast to Rowley 1999, no changes were made to the medications administrated to patients prior to ASCT.

Patients and those individuals in close contact with the patient such as family members and nursing staff were asked to complete a series of surveys to assess the impact of the washing protocol on their own well being.

Statistical Methods

The VASQOL responses along the 10 cm line were measured with a ruler with the beginning of the line equalling 0 and the data recorded in centrimetres. A number of statistical methods to analyse the data obtained from the VASQOL surveys. In order to correct for the skewed nature of the data, all the data (with the exception of the Wilcoxon rank sum test) underwent logarithmic transformation prior to analysis. Wilcoxon rank sum tests and

Figure 3.2

STEM CELL TRANSPLANT PATIENT SURVEY:
PRE-REINFUSION

Patient Name: _____ Date: _____

UR. No: _____ Time:_____ a.m./p.m.

DOB: _____

Please mark the line at the point that describes how <u>you personally</u> feel at this moment

1. NERVOUSNESS

Not at all _____ Extremely

2. FLUSHING

No flushing _____ The worst flushing I can imagine

3. NAUSEA

No nausea _____ The worst nausea I can imagine

3. UNUSUAL TASTES

Normal for me _____ The worst taste I can imagine

4. SHORTNESS OF BREATH

No shortness of breath. _____ The worst shortness of breath I can imagine

5. PAIN

No pain _____ The worst pain I can imagine

Location of pain:

<u>Comments:</u>

Figure 3.3

STEM CELL TRANSPLANT FAMILY MEMBER SURVEY:
PRE-REINFUSION

Name: _____ Date: _____

Time: _____ a.m./p.m.

Please mark the line at the point that describes how you personally feel at this moment

1. UNUSUAL SMELLS

Not at all _____ Extremely unpleasant

2. NAUSEA

No nausea _____ The worst nausea I can imagine

3. UNUSUAL TASTES

Normal for me _____ The worst taste I can imagine

4. SHORTNESS OF BREATH

No shortness of breath. _____ The worst shortness of breath I can imagine

5. PAIN

No pain _____ The worst pain I can imagine

Location of pain:

Comments:

Figure 3.4

STEM CELL TRANSPLANT NURSES SURVEY:

PRE REINFUSION

Name: _____ Date: _____

Time: _____ a.m./p.m.

Please mark the line at the point that describes how <u>you personally</u> feel at this moment

1. UNUSUAL SMELLS

Not at all _____ Extremely unpleasant

2. NAUSEA

No nausea _____ The worst nausea I can imagine

3. UNUSUAL TASTES

Normal for me _____ The worst taste I can imagine

4. SHORTNESS OF BREATH

No shortness of breath. _____ The worst shortness of breath I can imagine

5. PAIN

No pain _____ The worst pain I can imagine

Location of pain:

<u>Comments:</u>

Welch two sample t-tests were used to compare individual responses across treatment groups at the 24 hour mark [Zar, 1999]. A multivariate analysis of variance (MANOVA) using Wilk's lambda as a test statistic was used to compare responses from the two treatment groups at the 24 hour mark [Stevens, 2001]. Furthermore, the multivariate approach to repeated measures analysis was used to compare the profile of individual responses over time [Stevens, 2001]. Figure 3.5 demonstrates a summary of the strategy used for statistical analysis of the VASQOL data and highlights the findings. The criterion for significance was that a probability value of $P < 0.1$ indicated weak evidence, a probability value of $P < 0.05$ indicated moderate evidence while a probability value of $P < 0.01$ indicated strong evidence.

3.3 Results

3.3.1 Clinical Details of Patients

Temperature, blood pressure and urinary haemoglobin for all patients enrolled in the clinical trial were measured by nursing staff prior to and post ASCT. This was done in order to assess the impact of the washing protocol on patient clinical responses in comparison to our current practice.

Patient Temperature

Temperature readings measured prior to and post ASCT for patients enrolled in the clinical trial are shown in detail in Appendix A. Student's paired t-test analysis of the temperature readings demonstrated no significant difference in temperature for patients receiving *Washed* ($p = 0.216$, $n = 14$) or *Unwashed* ($p = 0.530$, $n = 31$)ASCT as displayed in Figure 3.6. This suggests that the results for the *Washed* group are consistent with the results of those patients receiving *Unwashed* HSC. Therefore, the washing protocol does not have an adverse affect on patient temperature and the results are comparable to current ASCT practise.

Patient Systolic Blood Pressure

Systolic blood pressure is measured during the period of ventricular contraction (systole) and is the higher of the two blood pressure readings. A systolic blood pressure between 90–120mmHg is defined as "normal" while hypertension is usually diagnosed on with a reading of 140mmHg or above [Anderson, 2002]. The blood pressure readings taken prior to and

Figure 3.5

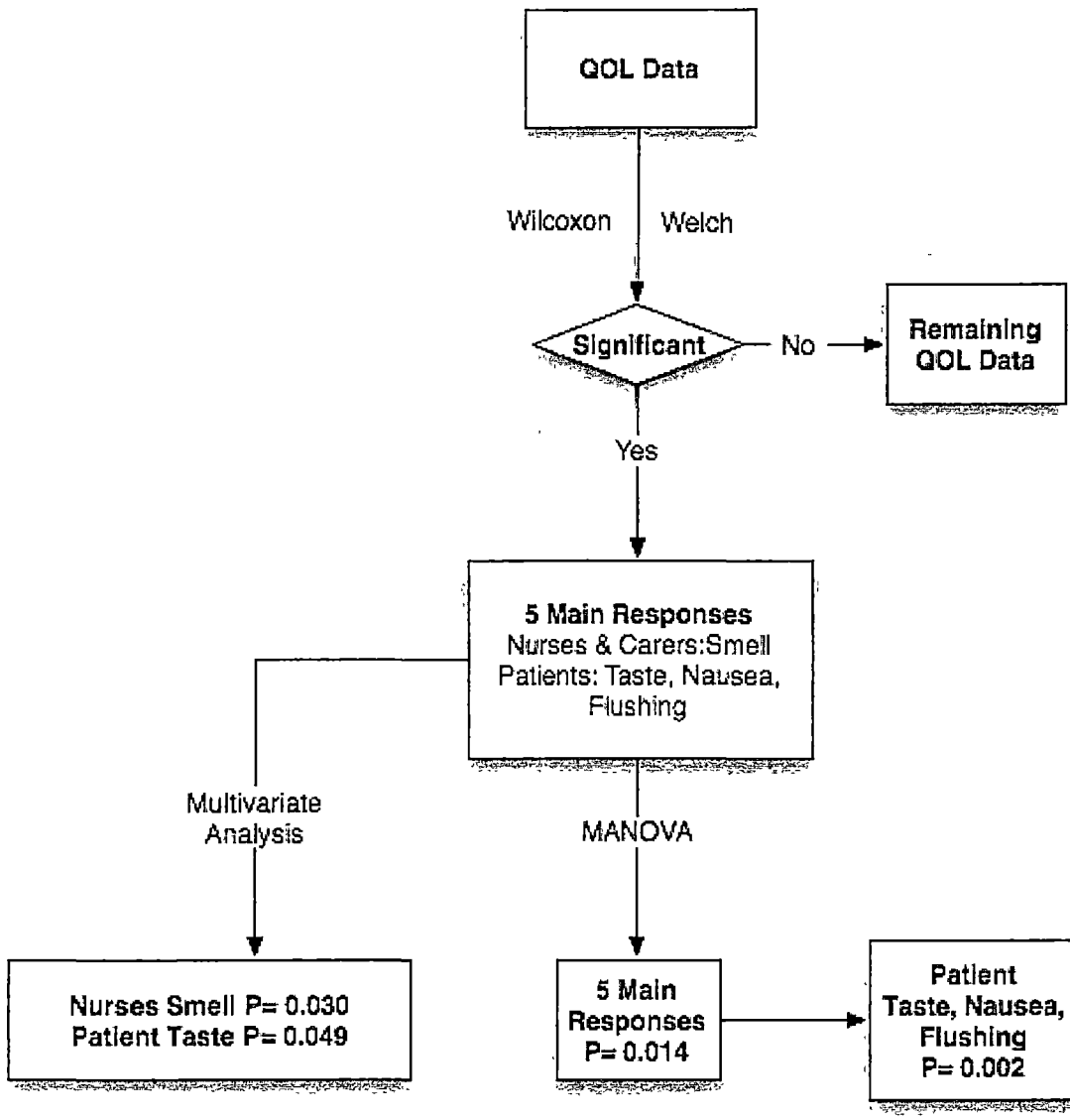


Figure 3.5: Summary of Statistical Analysis of VASQOL Data.

Flow chart summary of statistical analysis of VASQOL data obtained at 24 hour time point for patients, nurses and carers.

Figure 3.6

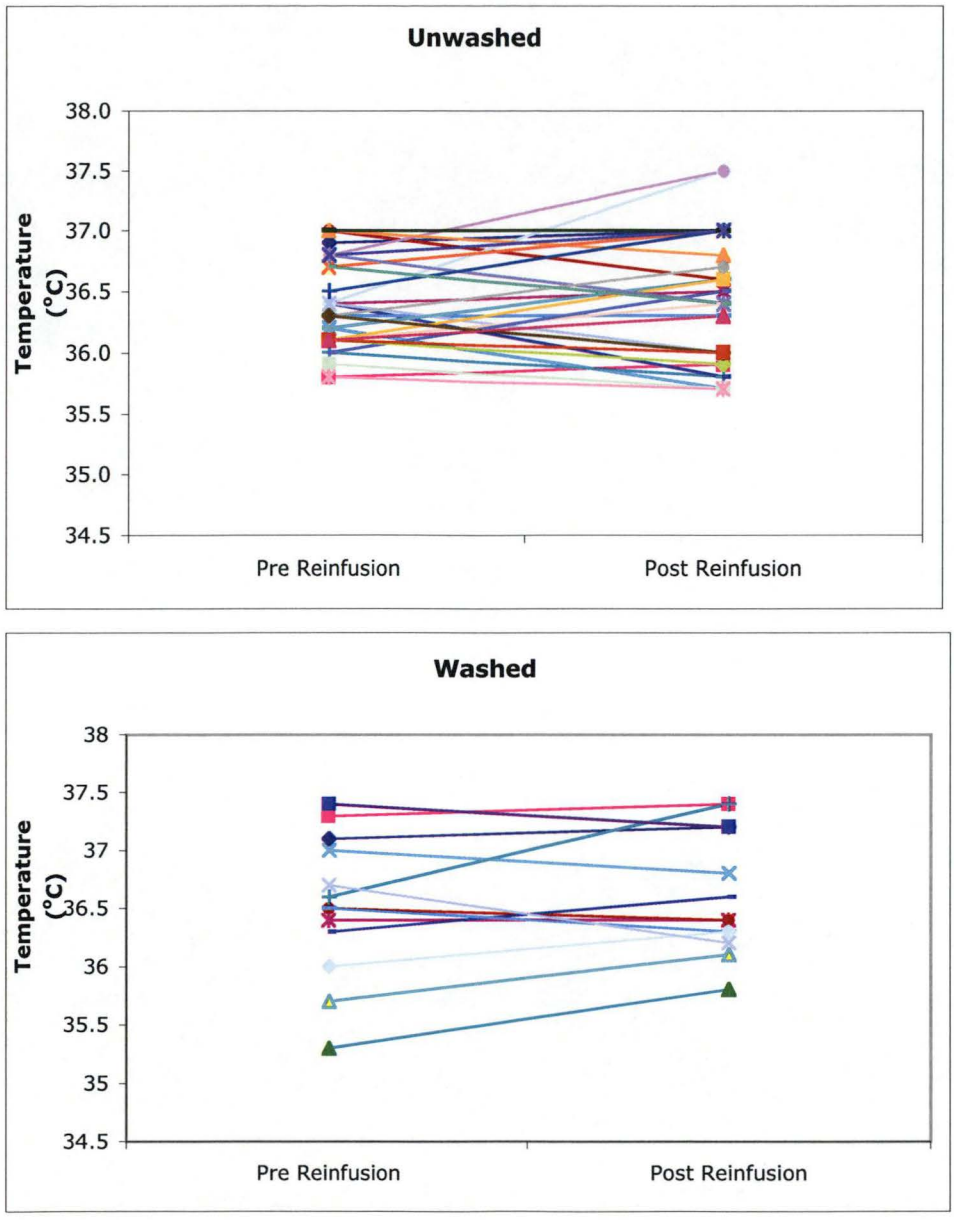


Figure 3.6:Comparison of Temperature of Patients Receiving Washed and Unwashed ASCT

Pre and Post ASCT patient temperatures are displayed for *Washed* and *Unwashed* ASCT as measured by nursing staff. There was no significant difference post reinfusion for patients receiving *Unwashed* ASCT post reinfusion ($p = 0.530$, $n = 31$) or *Washed* ASCT ($p = 0.216$, $n = 14$) for the patients receiving *Washed* ASCT.

post ASCT for patients enrolled in the clinical trial are shown in detail in Appendix A.

Student's paired t-test analysis of the systolic blood pressure readings demonstrated a significant difference between pre (range 97–161) and post (range 103–166) systolic blood pressure for patients receiving *Unwashed* ($p = 0.001$) ($n = 37$) ASCT as displayed in Figure 3.7. However, there was no significant difference between pre (range 101–135), and post reinfusion (range 102–136) ($n = 12$) for those patients receiving *Washed* ($p = 0.428$) ASCT. Therefore, the washing protocol is an advancement on current ASCT practice which demonstrated a significant relationship between the reinfusion of *Unwashed* ASCT and increased blood pressure.

However, if we examine the numbers of subjects that show an increase in the groups:

	Increase	No Increase
Washed	25	12
Unwashed	7	5

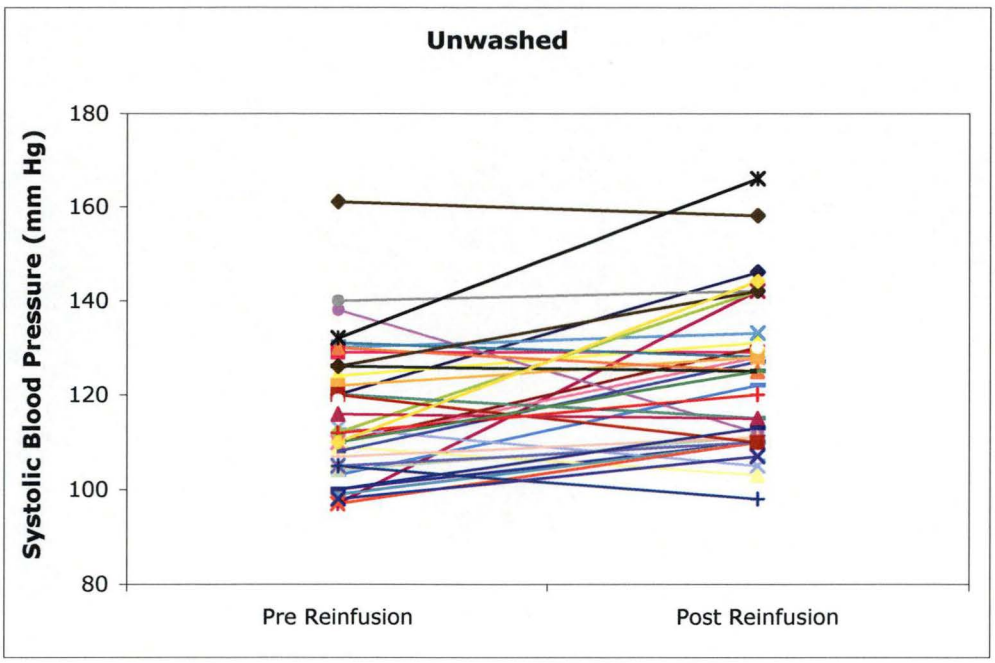
we observe that a greater fraction show an increase in the *Unwashed* group and a Fishers exact test shows no evidence (p= 0.73) of an association between treatment and BP increase. This may simply be a reflection of the lesser power of the Fisher exact test (alternatively contingency table analysis).

Patient Urinary Haemoglobin

Dipstick analysis (Hemastix, Bayer) was performed on patient urine obtained prior to and post ASCT, in order to measure free haemoglobin and the results (in red cell equivalent/μL) read after 60 seconds. Categories were assigned to haemoglobin levels as indicated in Appendix A on the basis of the presence (positive = 1) or absence (negative = 0) of urinary haemoglobin.

The percentage of patients who tested positive for urinary haemoglobin post reinfusion was 10.0% (1/10) for the *Washed* group compared to 27.6% (8/29) for *Unwashed* ASCT group. However, if the patients who tested positive for urinary haemoglobin prior to ASCT are excluded from analysis of the *Unwashed* ASCT group (4/24) this decreases to 16.7% as displayed in Figure 3.8. Thus, the washing protocol does not appear to have an adverse effect on patients in comparison to the *Unwashed* group, and may reduce the amount of free haemoglobin excreted by ASCT patients.

Figure 3.7



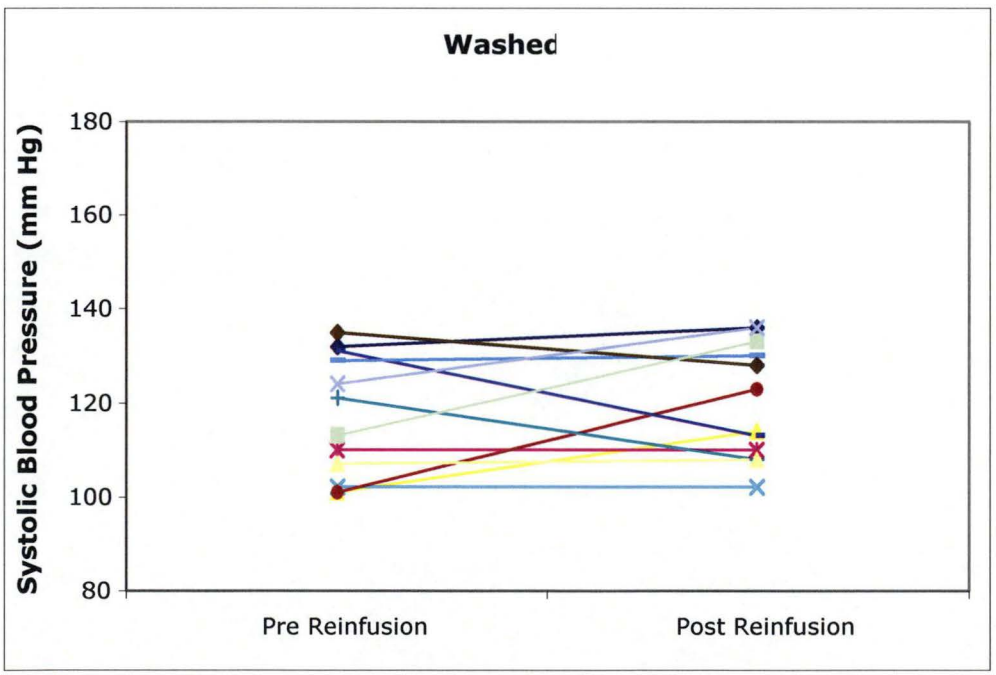


Figure 3.7:Comparison of Systolic Blood Pressure of Patients Receiving Washed and Unwashed ASCT

Pre and Post ASCT patient systolic blood pressures are displayed for *Washed* and *Unwashed* ASCT as measured by nursing staff. There is a significant difference post reinfusion for patients receiving *Unwashed* ASCT post reinfusion ($p = 0.001$, $n = 37$), whereas there was no difference post reinfusion ($p = 0.428$, $n = 12$) for the patients receiving *Washed* ASCT.

Figure 3.8

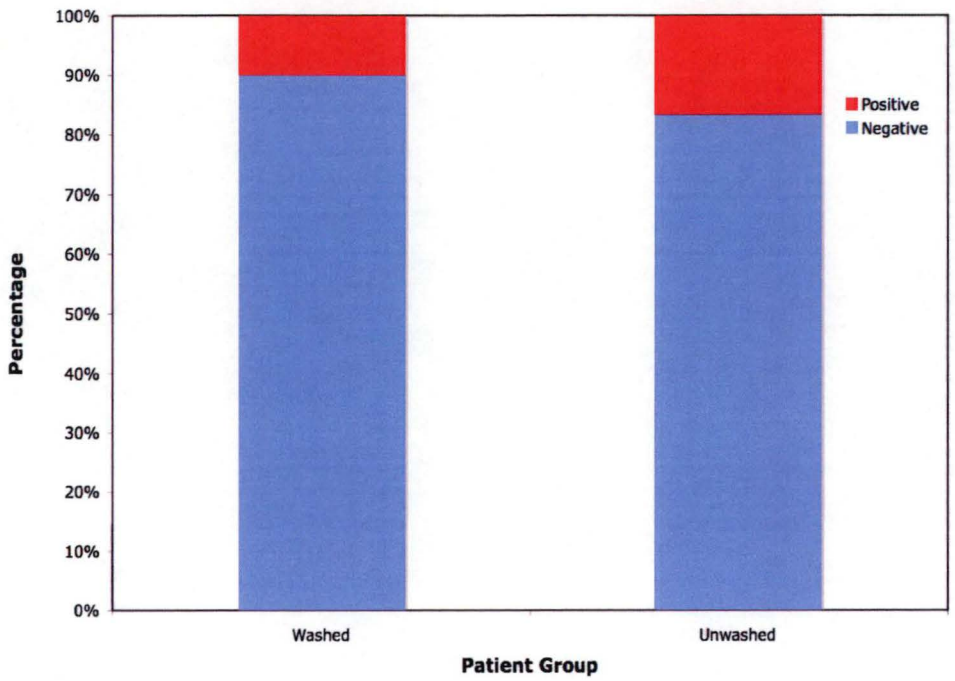


Figure 3.8:Comparison of the Post ASCT Urinary Haemoglobin Percentage Results of Patients Receiving Washed and Unwashed ASCT

Patient urinary haemoglobin was measured using a dipstick test post ASCT by nursing staff and results were recorded as positive or negative. Results shown for *Unwashed* are of 24 patients and *Washed* are for 10 patients expressed as percentage of subject group.

Patient No.	Microbiology
1	No Growth
2	Positive Growth[1]
3	No Growth
4	Positive Growth[1]
5	No Growth
6	No Growth
7	No Growth
8	No Growth
9	No Growth
10	No Growth
11	No Growth
12	No Growth
13	No Growth
14	No Growth
15	No Growth
16	No Growth
17	No Growth
18	No Growth
19	No Growth

Table 3.4: Washed HSC Microbiology Results

3.3.2 Microbiology Testing

Samples for anaerobic (Bactec Plus+) and aerobic (Bactec Peds Plus/F aerobic) microbiological testing were taken from the expressed supernatant of all patients undergoing reinfusion with *Washed* HSC as displayed in Table 3.4.

As the only two positive bacterial growth reports were from cryobags which had been damaged during storage in liquid nitrogen, it is possible that these cryobags may have been exposed to contamination prior to post-thaw washing. Furthermore, as 89.5% of the samples tested (17/19) reported no growth it appears that possibility of microbiological contamination of the HSC as a result of the washing protocol is limited. Therefore, the testing of supernatant is a useful quality control measure that validates the safety of the washing protocol in relation to bacterial contamination. The decision to use damaged cryobags in preference to undamaged bags remaining in storage was made by the consulting physician on the basis of HSC dosage.

[1]Patient cryobags damaged during storage in liquid nitrogen.

Table 3.5

Unwashed Patient QOL Summary

Pre Reinfusion

	Flushing	Unusual Tastes	Nausea	S.O.B	Pain	Nervousness
Ave.	0.5	1.3	2.3	0.4	0.5	2.7
SD	1.4	2.2	3.1	1.0	1.3	2.6
Median	0.0	0.0	0.5	0.0	0.0	2.1

Post Reinfusion

	Flushing	Unusual Tastes	Nausea	S.O.B	Pain	Nervousness
Ave.	1.1	2.7	1.4	0.5	0.7	1.6
SD	2.1	3.0	2.5	1.3	1.8	2.8
Median	0.0	1.9	0.0	0.0	0.0	0.0

At Time of Discharge (or 6 Hours Post Reinfusion)

	Flushing	Unusual Tastes	Nausea	S.O.B	Pain	Nervousness
Ave.	0.4	2.0	1.9	0.7	0.5	0.6
SD	0.9	2.2	2.5	1.5	1.0	1.2
Median	0.0	1.5	0.7	0.0	0.0	0.0

24 Hours Post Reinfusion

	Flushing	Unusual Tastes	Nausea	S.O.B	Pain	Nervousness
Ave	0.2	2.7	3.4	0.8	0.9	0.9
SD	0.4	2.9	3.0	1.5	1.7	1.8
Median	0.0	2.3	3.1	0.0	0.0	0.0

	Volume (mls)
Ave.	148.6
SD	100.7
Median	94.0

Table 3.6

Washed Patient QOL Summary

Pre Reinfusion

	Flushing	Unusual Tastes	Nausea	S.O.B	Pain	Nervousness
Ave.	0.7	0.9	1.8	0.3	0.6	1.0
SD	1.6	1.2	1.8	0.5	1.5	1.1
Median	0.0	0.3	1.4	0.0	0.0	1.0

Post Reinfusion

	Flushing	Unusual Tastes	Nausea	S.O.B	Pain	Nervousness
Ave.	1.1	1.3	1.3	0.3	0.7	0.4
SD	1.5	2.1	2.1	0.5	1.0	0.7
Median	0.4	0.6	0.3	0.0	0.0	0.1

At Time of Discharge (or 6 Hours Post Reinfusion)

	Flushing	Unusual Tastes	Nausea	S.O.B	Pain	Nervousness
Ave.	1.1	1.1	1.9	0.4	0.9	0.2
SD	1.6	1.2	2.5	0.5	1.4	0.4
Median	0.2	0.7	0.7	0.2	0.4	0.0

24 Hours Post Reinfusion

	Flushing	Unusual Tastes	Nausea	S.O.B	Pain	Nervousness
Ave	0.7	0.9	2.1	0.6	0.7	0.3
SD	0.9	1.2	2.3	1.0	1.4	0.5
Median	0.2	0.5	1.7	0.0	0.0	0.0

	Volume(mls)
Ave.	116.8
SD	58.0
Median	97.0

Figure 3.9

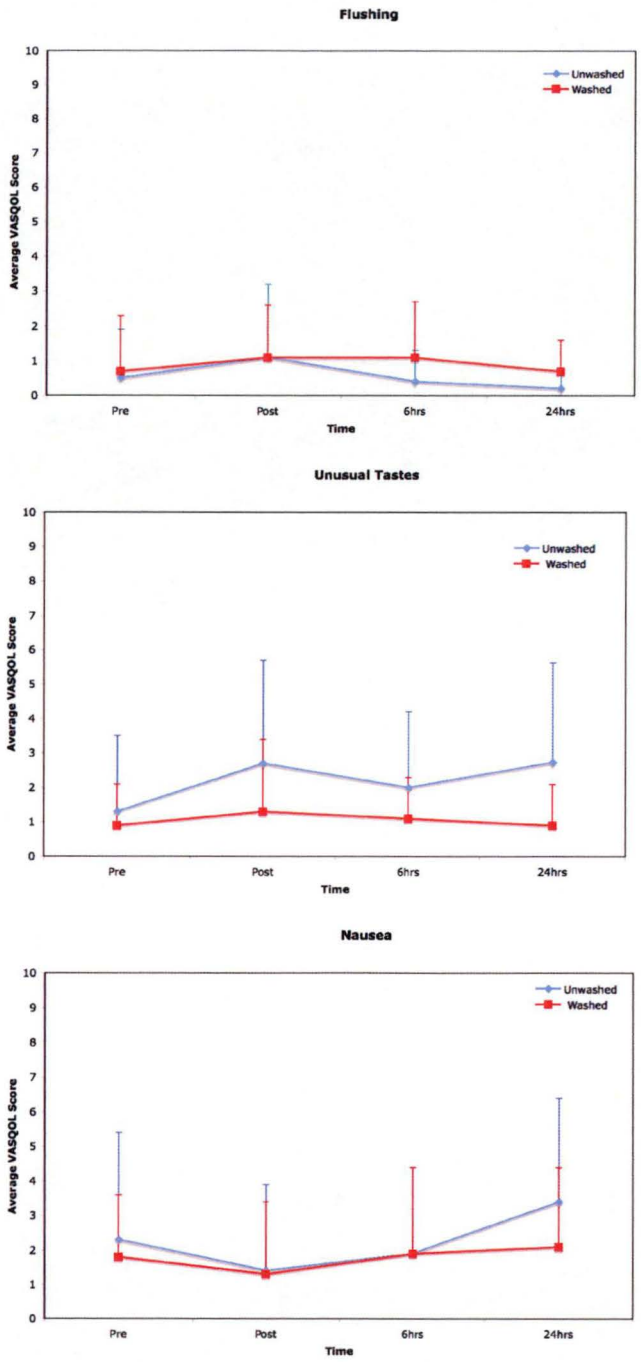


Figure 3.9: Comparison of Average VASQOL Scores of Symptoms Experienced by Patients Receiving Washed and Unwashed ASCT

VASQOL scores for symptoms associated with ASCT were assigned over a 24 hour period by patients receiving *Washed* and *Unwashed* ASCT. Results shown for *Unwashed* are of 43 patients and *Washed* are for 19 patients.

3.3.3 VASQOL surveys

Prior to statistical analysis a preliminary analysis of the raw VASQOL data for patients, carers and nurses at each time point in the 24 hour period (shown in detail in Appendix B) was performed in order to determine if there were any trends in the observations for flushing, tastes, nausea, smells, shortness of breath, pain and nervousness. All average VASQOL results of less than 1.0 were excluded from further exploratory comment given the proximity of these values to zero on the VASQOL 10cm linear scale which signifies no response therefore subjects didn't exhibit these symptoms. The findings of the preliminary analysis provided guidance for the more detailed statistical analysis of all the VASQOL data.

Exploratory Analysis

Examination of the average VASQOL data at each time point over the 24 hour period suggests that regardless of whether individuals were involved with *Unwashed* or *Washed* HSC that the patients (Tables 3.5 and 3.6) nurses (Tables 3.7 and 3.8)and carers (Tables 3.9 and 3.10) did not routinely experience shortness of breath and pain following ASCT. Additionally, nausea and unusual tastes were not regularly reported by nurses and carers following ASCT. Therefore these symptoms may only an occasional issue for individuals involved with ASCT.

Patients who received *Unwashed* HSC recorded an average VASQOL of 1.1 (SD 2.1) for flushing post reinfusion, however this declines to below 1.0 for subsequent readings (Table 3.5). These results are comparable with *Washed* ASCT patients (Table 3.6). As the average VASQOL values for both the *Unwashed* and *Washed* groups are close to the cut off value throughout the 24 hour period, flushing appears to be only a minor issue to patients following ASCT (Figure 3.9).

The average VASQOL scores for unusual tastes remain above 1.0 for *Unwashed* patients throughout the survey period suggesting that this symptom is a concern for patients receiving *Unwashed* HSC (Table 3.5, Figure 3.9). In comparison, the average VASQOL values for the *Washed* patient group are close to the cut off value throughout the 24 hour period nausea Table 3.6)(Figure 3.9). This suggests that unusual tastes are a lesser concern for those patients who received *Washed* HSC and is an improvement on current ASCT practise.

Nausea following ASCT is a concern for patients with average VASQOL values remaining above 1.0 for both the *Unwashed* and *Washed* patient groups throughout the 24 hour period (Tables 3.5 and 3.6) (Figure 3.9).

However, it is worth noting that the average pre reinfusion VASQOL values for nausea were greater than 1.0 for both the *Unwashed* and *Washed* groups (2.3 SD3.1 and 1.8 SD 1.8 respectively).

The only symptom to receive average VASQOL values of greater than 1.0 following reinfusion (as observed by nurses involved in *Unwashed* ASCT) was unusual smells (Table 3.7). However, by the 24 hour time point the average VASQOL value had decreased by approximately 50% to 1.1 (SD2.2) suggesting that the intensity of the smell was diminishing as the DMSO was excreted by the patients (Figure 3.10). Nurses involved in *Washed* ASCT also recorded average VASQOL values of greater than 1.0 post reinfusion which decreased over time (Table 3.8). By the 24 hour time point the average VASQOL score had decreased to 0.0 (SD0.1). This suggests that the intensity of the smell associated with *Washed* ASCT diminished at a faster rate than experienced by nurses associated with *Unwashed* ASCT.

Similar to the nurses, unusual smells was also the only symptom observed by carers involved to receive average VASQOL values of greater than 1.0 at all time points post reinfusion for *Unwashed* ASCT (Table 3.9). This suggests that unusual smells remain a concern for carers throughout the 24 hour period following *Unwashed* ASCT. Carers associated with *Washed* ASCTs reported average VASQOL values of 1.8 (SD1.5) and 2.5 (SD2.4) at the post reinfusion and 6 hours time points. However, by the 24 hour time point the average VASQOL had decreased to 0.3 suggesting that the smell was no longer a concern to carers which is in contrast to the experience of carers associated with *Unwashed* ASCT (Figure 3.11).

The average volume reinfused in the *Washed* arm was 116.8mls (SD 58.0) (Table 3.6), while the average volume reinfused in the *Unwashed* arm of the clinical trial were 148.6 mls (SD 100.7) (Table 3.5). However, as the median volumes received by patients in the *Unwashed* arm of the clinical trial was 94.0 mls (Table 3.5) and 97.0 mls (Table 3.6) for patients enrolled in the *Washed* arm are comparable, this suggests that the differences in average volumes reinfused is due to a few larger volume reinfusions rather than consistent differences in volumes (see Appendix B) between the *Washed* and *Unwashed* arms.

Statistical analysis

Preliminary analysis of the VASQOL responses for symptoms previously attributed to ASCT [Rowley et al., 1999a] suggests that unusual tastes, nausea and flushing seem to be of most concern for patients, while unusual smells were the main concern for nurses and carers therefore they will be the primary focus of the statistical analysis.

Table 3.7

Unwashed Nurses QOL Summary

Pre Reinfusion

	Smells	Unusual Tastes	Nausea	S.O.B	Pain
Ave.	0.1	0.0	0.1	0.0	0.1
SD	0.5	0.0	0.5	0.1	0.4
Median	0.0	0.0	0.0	0.0	0.0

Post Reinfusion

	Smells	Unusual Tastes	Nausea	S.O.B	Pain
Ave.	2.2	0.3	0.3	0.0	0.1
SD	1.7	0.8	0.7	0.1	0.2
Median	1.8	0.0	0.0	0.0	0.0

At Time of Discharge (or 6 Hours Post Reinfusion)

	Smells	Unusual Tastes	Nausea	S.O.B	Pain
Ave.	2.3	0.4	0.1	0.2	0.1
SD	2.5	1.7	0.6	0.9	0.3
Median	1.7	0.0	0.0	0.0	0.0

24 Hours Post Reinfusion

	Smells	Unusual Tastes	Nausea	S.O.B	Pain
Ave.	1.1	0.1	0.1	0.0	0.0
SD	2.2	0.3	0.2	0.2	0.1
Median	0.0	0.0	0.0	0.0	0.0

Table 3.8

Washed Nurse QOL Summary

Pre Reinfusion

	Smells	Unusual Tastes	Nausea	S.O.B	Pain
Ave.	0.1	0.0	0.0	0.0	0.1
SD	0.3	0.1	0.1	0.1	0.3
Median	0.0	0.0	0.0	0.0	0.0

Post Reinfusion

	Smells	Unusual Tastes	Nausea	S.O.B	Pain
Ave.	2.2	0.0	0.1	0.0	0.1
SD	2.8	0.1	0.2	0.1	0.3
Median	1.1	0.0	0.0	0.0	0.0

At Time of Discharge (or 6 Hours Post Reinfusion)

	Smells	Unusual Tastes	Nausea	S.O.B	Pain
Ave.	1.3	0.1	0.0	0.0	0.2
SD	1.9	0.2	0.1	0.2	0.3
Median	0.1	0.2	0.1	0.1	0.2

24 Hours Post Reinfusion

	Smells	Unusual Tastes	Nausea	S.O.B	Pain
Ave.	0.0	0.0	0.0	0.0	0.2
SD	0.1	0.1	0.0	0.1	0.5
Median	0.0	0.0	0.0	0.0	0.0

Figure 3.10

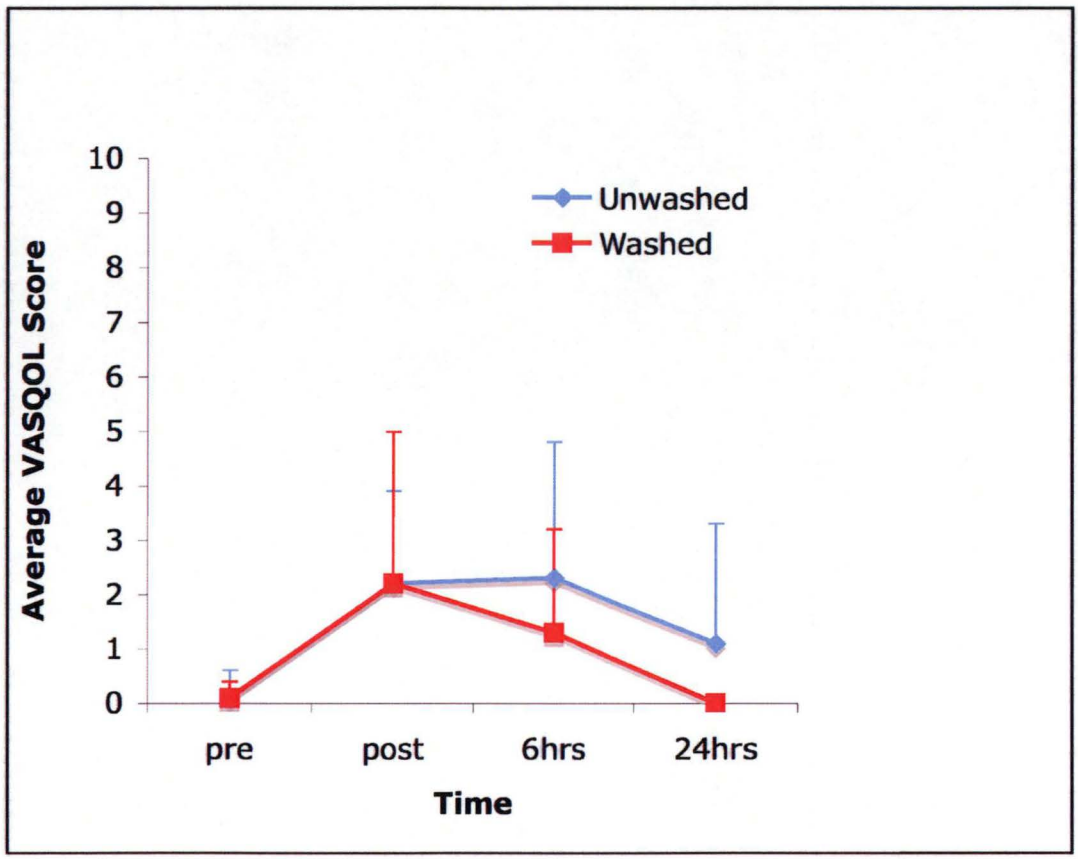


Figure 3.10: Comparison of Average VASQOL Scores of Unusual Smells Experienced by Nurses Exposed to Washed and Unwashed ASCT

VASQOL scores for unusual smells associated with ASCT were assigned over a 24 hour period by nurses to exposed to *Washed* and *Unwashed* ASCT. Results shown for *Unwashed* are of 43 nurses and *Washed* are for 16 nurses.

Table 3.9

Unwashed Carer QOL Summary

Pre Reinfusion

	Smells	Unusual Tastes	Nausea	S.O.B	Pain
Ave.	0.0	0.1	0.4	0.1	0.0
SD	0.0	0.3	1.0	0.1	0.1
Median	0.0	0.0	0.0	0.0	0.0

Post Reinfusion

	Smells	Unusual Tastes	Nausea	S.O.B	Pain
Ave.	2.2	0.3	0.3	0.1	0.0
SD	3.0	1.0	1.0	0.1	0.1
Median	0.7	0.0	0.0	0.0	0.0

At Time of Discharge (or 6 Hours Post Reinfusion)

	Smells	Unusual Tastes	Nausea	S.O.B	Pain
Ave.	3.9	0.0	1.4	0.1	0.0
SD	3.5	1.5	2.1	0.2	0.1
Median	2.4	0.0	0.0	0.0	0.0

24 Hours Post Reinfusion

	Smells	Unusual Tastes	Nausea	S.O.B	Pain
Ave.	2.7	0.1	0.1	0.1	0.1
SD	3.4	0.2	0.3	0.3	0.2
Median	1.2	0.0	0.0	0.0	0.0

Table 3.10

Washed Carer QOL Summary

Pre Reinfusion

	Smells	Unusual Tastes	Nausea	S.O.B	Pain
Ave.	0.2	0.0	0.2	0.1	0.0
SD	0.7	0.2	0.7	0.2	0.1
Median	0.0	0.0	0.0	0.0	0.0

Post Reinfusion

	Smells	Unusual Tastes	Nausea	S.O.B	Pain
Ave.	1.8	0.2	0.0	0.1	0.1
SD	1.5	0.7	0.1	0.2	0.3
Median	1.7	0.0	0.0	0.0	0.0

At Time of Discharge (or 6 Hours Post Reinfusion)

	Smells	Unusual Tastes	Nausea	S.O.B	Pain
Ave.	2.5	0.1	0.4	0.1	0.1
SD	2.4	0.1	1.0	0.2	0.2
Median	1.8	0.0	0.0	0.0	0.0

24 Hours Post Reinfusion

	Smells	Unusual Tastes	Nausea	S.O.B	Pain
Ave.	0.3	0.2	0.2	0.2	0.1
SD	0.6	0.4	0.5	0.5	0.2
Median	0.0	0.0	0.0	0.0	0.0

Figure 3.11

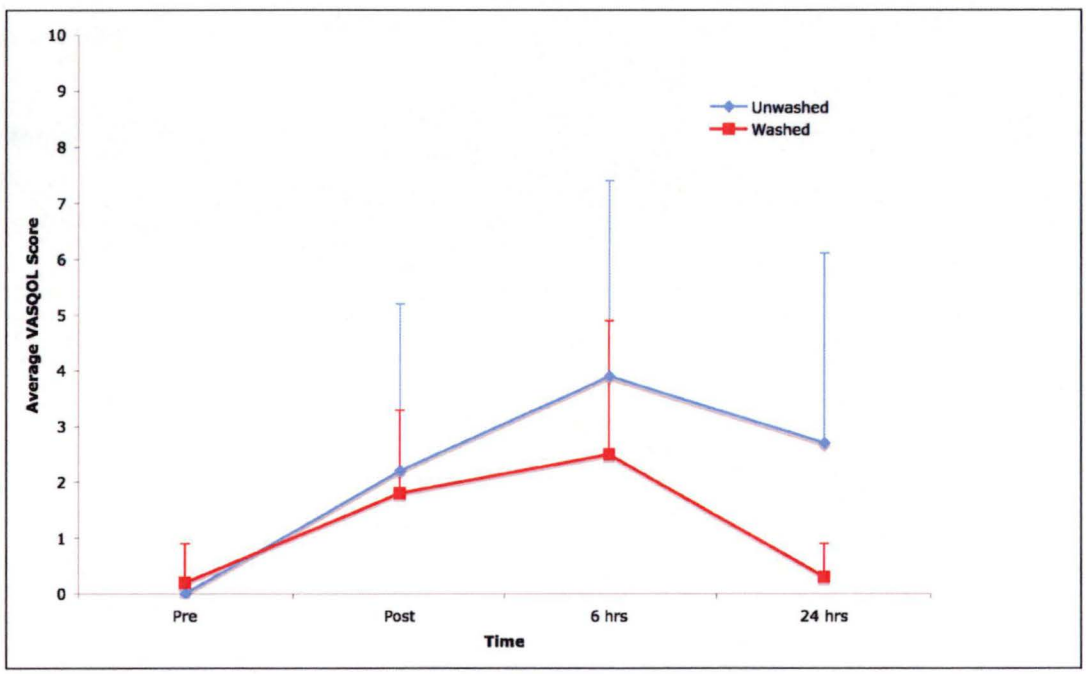


Figure 3.11: Comparison of Average VASQOL Score of Unusual Smells Experienced by Carers Exposed to Washed and Unwashed ASCT

VASQOL scores for unusual smells associated with ASCT were assigned over a 24 hour period by carers to exposed to *Washed* and *Unwashed* ASCT. Results shown for *Unwashed* are of 26 carers and *Washed* are for 17 carers.

Table 3.11 Comparison of p Values of VASQOL Responses for *Washed* and *Unwashed* Groups at 24 hour time point

	Carers		Nurses		Patients	
Symptom	Welch	Wilcoxon	Welch	Wilcoxon	Welch	Wilcoxon
Smell	0.021	0.064	0.003	0.059	...	...
Taste	0.335	0.513	0.360	0.886	0.062	0.077
Nausea	0.407	0.415	0.210	0.861	0.072	0.049
SOB	0.761	0.767	0.878	0.871	0.670	0.931
Pain	0.703	0.813	0.984	0.871	0.697	0.922
Flushing	...	...	...	...	0.047	0.023
Nervousness	...	...	...	...	0.095	0.606

The variability of data obtained from the VASQOL surveys (shown in detail in Appendix B) influenced the choice of statistical methods required for analysis. Extensive graphical examination showed no indication that particular nurses were responsible for the observed differences in scores. Many of the responses were close to zero, however there were also several extreme responses which produce skewed data and increase the overall mean and SD. Therefore, with the exception of the Wilcoxon rank sum test, logarithmic transformation of the VASQOL data was necessary prior to statistical analysis to correct for the skewed nature of the data [Azcel, 1999].

Recall that the criterion for significance was that a probability value of $P < 0.1$ indicated weak evidence, a probability value of $P < 0.05$ indicated moderate evidence while a probability value of $P < 0.01$ indicated strong evidence.

Table 3.11 shows the results of the Welch t-tests and the Wilcoxon rank sum tests applied to all of the data collected at the 24 hour time point. As the majority of our ASCTs are performed as an outpatient procedure, the 24 hour time point were chosen for statistical analysis as this correlates with the first post transplant evaluation of patients following their return in our outpatient facility for monitoring. Although the Welch test shows strong evidence, the more conservative Wilcoxon test shows moderate evidence of a treatment difference between *Washed* and *Unwashed* responses for smell from both carers ($p = 0.021$, $p = 0.064$) and nurses ($p = 0.003$, $p = 0.059$) perspective. Furthermore, both the tests also support the preliminary analysis observations of a treatment difference between *Washed* and *Unwashed* responses for nausea ($p = 0.072$, $p = 0.049$), flushing ($p = 0.047$, $p = 0.023$) and tastes ($p = 0.062$, $p = 0.077$) for patients at the 24 hour mark.

The patient responses relating to nausea, unusual tastes and flushing as

Table 3.12 Comparison of MANOVA analysis for *Washed* and *Unwashed* Groups at 24 hour time point

Carers & Nurses & Patients		Patients	
Wilks Lambda	p Value	Wilks Lambda	p Value
0.480	0.014	0.723	0.002

well as responses from nurses and carers relating to unusual smells were of primary interest. Shortness of breath, pain and nervousness for all individuals as well as taste and nausea for nurses and carers were also analysed for completeness which showed no significant differences between the *Washed* and *Unwashed* groups. Therefore further detailed analysis will be confined to smell responses for nurses and carers as well as nausea, flushing and taste for patients.

At the 24 hour point the results of the MANOVA analysis as applied to these five responses of interest indicates a significant difference between the *Washed* and *Unwashed* responses as shown in Table 3.12 (Wilks lambda $= 0.480$ and $p = 0.014$). However, this analysis requires a complete set of data and if any of the responses were missing, as was often the case for the smell response from both the nurses and carers, the entire case must be deleted from the analysis, reducing the strength of the analysis. Figure 3.12 shows QQ plots [Stevens, 2001] of the residuals from the fit for both the combined responses and of just the patient responses. The QQ plots of the residuals from the fit for the combined response suggests that the data is not normally distributed and remains skewed even after log transformation casting some doubt on the reliability of the MANOVA analysis.

The results of the same MANOVA analysis restricted to just the three responses from the patients at 24 hours displayed in Table 3.12., demonstrates that washing has a significant effect on the patient responses (Wilks lambda $= 0.723$ and $p = 0.002$). Additionally, the QQ plots of just the patient responses (Figure 3.12) were improved in comparison, as more data sets were used. Therefore, the MANOVA analysis of just the patient responses is more reliable and demonstrates a treatment difference between the *Washed* and *Unwashed* responses, indicating an improvement on the current ASCT procedure.

Multivariate analysis of the 5 main VASQOL responses for all time points is outlined in Table 3.13. In this analysis the changes in response over time were analysed with a multivariate analysis of variance to establish if there was a change ("shift") in the overall group mean as well as if there was any differences between the responses over time ("parallelism"). As was the case

Figure 3.12

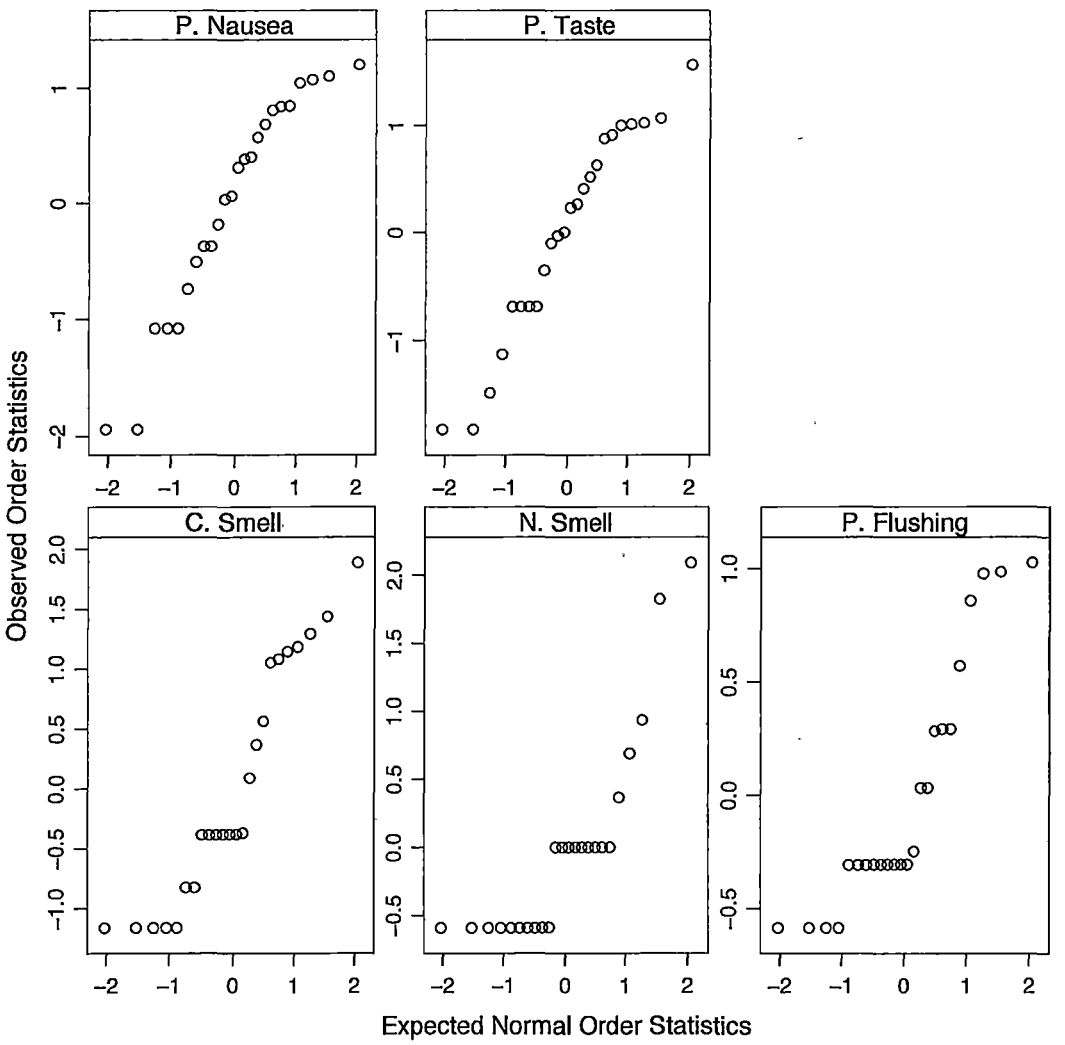


Figure 3.12: QQ Plots of the Five Main Responses for all Subjects at the 24 Hour Time Point.

QQ plots of VASQOL scores from patients for unusual tastes, flushing, nausea and unusual smells for both nurses and carers associated with ASCT at the 24 hour point.

Table 3.13 Comparison of Multivariate Analysis of VASQOL Responses p values for *Washed* and *Unwashed* Groups at all time points

	Carers		Nurses		Patients	
Symptom	parallelism	mean shift	parallelism	mean shift	parallelism	mean shift
Smell	0.280	0.364	0.076	0.030	...	...
Taste	...	...	...	...	0.263	0.049
Nausea	...	...	...	...	0.491	0.243
Flushing	...	...	...	...	0.343	0.263

for the MANOVA if any response was missing, then all observations for that case were ignored. The majority of the responses do not demonstrate any significant difference as a consequence of the washing protocol over time. However, there is evidence of a treatment difference for both nurses smells ($p = 0.030$) and patient tastes ($p = 0.049$) for all time points. This suggests that the washing protocol reduced the impact of unusual tastes and smells for patients and nurses respectively over the 24 hour time period.

3.4 Discussion

The potential to reduce re-infusional toxicity associated with ASCT by the safe post-thaw removal of DMSO and cellular debris has been a possibility since the NYCBB reported improved recovery of viable cryopreserved umbilical cord blood HSC following washing with isotonic solution [Rubinstein et al., 1995]. In addition, the impact of post-thaw washing of upon patient recovery has been successfully investigated with regard to patient engraftment [Nagamura-Inoue et al., 2003, Syme et al., 2004]. However, little research has been conducted from the patient perspective in order to determine the extent of the reduction in side effects associated with the reinfusion of HSC. This thesis reports on the findings of a clinical trial to determine if the procedure is of significant benefit to patients.

The measurement of ASCT patient physical observations pre and post reinfusion by nursing staff provides valuable insight into the impact of washing HSC on patient well-being. This study demonstrated a significant correlation between the reinfusion of *Unwashed* ASCT and elevated blood pressure, which is consistent with previous findings [Kessinger et al., 1990, Davis et al., 1990, Stroncek et al., 1991]. Possible causes of hypertension related to ASCT include the effect of DMSO on cardiovascular or smooth muscles as well as the infusion of cell lysis products [Davis et al., 1990]. In contrast, the reinfusion of *Washed* HSC does not have an adverse effect on

patient blood pressure. Hence, washing HSC post-thawing reduces a potential cause of hypertension for ASCT patients and the associated complications including severe headaches, blurred vision, myocardial infarction or cerebrovascular accident [Anderson, 2002]. Therefore, it is an improvement on the reinfusion of unwashed HSC which has a significant correlation with increased blood pressure.

The washing protocol did not have any effect on patient temperature and the results are comparable to current ASCT practise. Moreover, as all of the samples from *Washed* supernatants taken from undamaged cryobags did not demonstrate any evidence of bacterial growth, the washing procedure does not appear to increase the risk of bacterial contamination. This is an important point from a Good Manufacturing Process (GMP) perspective as the extra manipulation due to washing does not increase manufacturing risk.

Testing of urine for free haemoglobin demonstrated a slight reduction in the percentage of *Washed* ASCT patients who tested positive for urinary haemoglobin post reinfusion in comparison to the *Unwashed* ASCT patient group. Haemoglobinuria has often been reported as a possible side effect of ASCT [Styler et al., 1992, Rowley et al., 1999a] with one previous study reporting that of 100 ASCT patients, 92% experienced haemoglobinuria and 80% experienced red urine post reinfusion of cryopreserved HSC [Kessinger et al., 1990]. Therefore the comparable results for free haemoglobin in our clinical trial of the *Unwashed* and *Washed* ASCT patient groups suggests that current HSC collection and storage strategies have already reduced the amount of red cell lysis products reinfused into patients during ASCT. However, in the event of large volume reinfusions, a protocol to reduce urinary haemoglobin by approximately 90% as validated by the laboratory study, remains an advantage.

Possible causes for the five patients who tested positive for urinary haemogloblin pre ASCT include damage to the epithelial cells from chemotherapy, kidney disease, urinary tract infection, bladder inflammation, prostrate gland enlargement, kidney stones or cancer of the prostate, bladder or kidneys [Anderson, 2002] [Gertz et al., 2004].

A number of problems with VASQOL surveys became apparent during the clinical trial including the recruitment of patients, subject compliance in the completion and return of surveys as well as analysis of data, all of which have been previously documented [Cella and Tulsky, 1993] [Cella, 1996]. Therefore, a number of strategies were instigated to counter these problems. In order to improve recruitment of subjects it was essential to initiate direct contact with the transplant co-ordinator and consultant physicians, informing them of all potential candidates for the clinical trial. Furthermore, to improve patient compliance it was necessary to educate all individuals involved

(patients, carers, medical consultants and nursing staff) about the VASQOL survey and to work in conjunction with the transplant co-ordinator to followup all participants in the clinical trial.

As VASQOL observations are subjective, there is huge variability in responses between individuals which makes for challenging data analysis. A possible strategy to reduce variability between subjects in future studies could be the use of a fixed set of observers throughout the VASQOL trial (i.e. nurses). Despite these issues, the data obtained from the VASQOL surveys provide an invaluable opportunity for subjective evaluation of the symptoms associated with the use of *Washed* and *Unwashed* HSC in ASCT from the perspective of the patients as well as nurses and carers.

Exploratory analysis of the VASQOL responses for all the symptoms previously associated with reinfusion (flushing, nausea, shortness of breath, pain, unusual smells and tastes) [Rowley et al., 1999a] suggested that the most commonly reported side effect following ASCT for patients was unusual tastes, although nausea and flushing were also concerns. In addition, unusual smells were the most commonly reported ASCT side effect for both the carers and nurses. The average VASQOL scores for the *Washed* and *Unwashed* groups suggested all of those individuals (patients, nurses and carers) involved with *Washed* ASCT experienced a faster rate of recovery from these ASCT related side effects.

As a result of the restrictions of this non-randomised clinical trial, it is also possible that any differences between the *Washed* and *Unwashed* results are related to differences in the sample size and reinfusion volumes of the respective treatment groups. However, the results do not demonstrate a clear relationship between reinfusion volume and VASQOL response i.e. for every large volume reinfusion that triggers a substantial response there is a large volume reinfusion that elicits a negligible response (as displayed in Figure 3.13.). Accordingly, it is likely that reinfusion volume is only one of a number of potential variables and that some individuals may be more sensitive to ASCT related side effects than others (e.g patients with a smaller body mass, or have experienced recent vomiting or nausea [Synder et al., 2004]).

As a consequence of the findings of the exploratory analysis, the Wilcoxon rank sum and Welch t-test tests were applied to the VASQOL responses of patient flushing, nausea and unpleasant tastes, as well as unusual smells as reported by carers and nurses at the 24 hour time point. The Welch t-test is a test of equality of two population distributions and is useful in testing for the equality of two population means. In comparison, the two-sample Wilcoxon rank sum test is used when the assumption of normal population distributions is not met and is slightly stronger than the Wilcoxon test [Azcel, 1999]. Both these tests showed weak to moderate evidence of treatment differences for

Figure 3.13

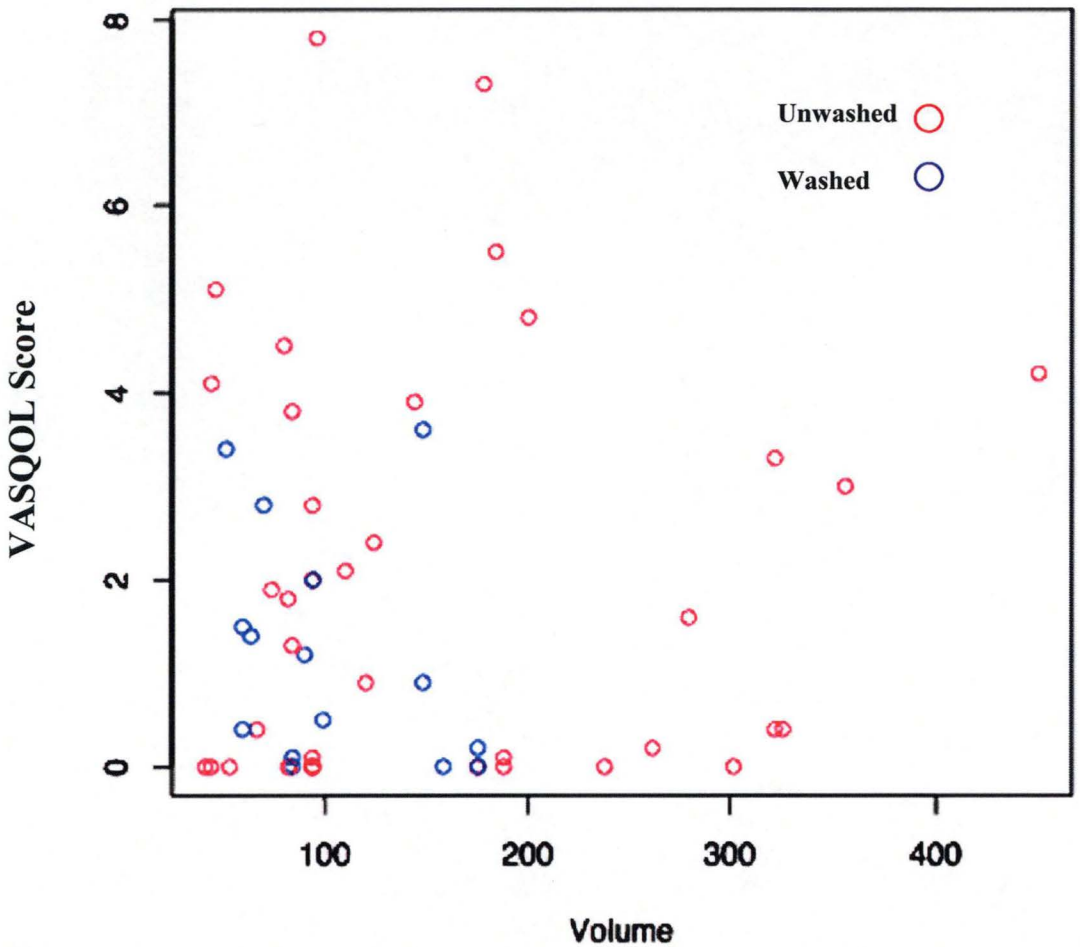


Figure 3.13: VASQOL Scores of Unusual Tastes Experienced by Patients versus Volume for Washed and Unwashed ASCT at 6 hour time point.

VASQOL scores for unusual tastes associated with ASCT were assigned over a 24 hour period by patients exposed to *Washed* and *Unwashed* ASCT. Results shown for *Unwashed* are of 43 patients and *Washed* are for 16 patients.

the patient symptoms of flushing, nausea and taste at the 24 hour time point. There is also weak to moderate evidence of treatment difference for smells as experienced by carers. Furthermore there is moderate to strong evidence of a difference for the smells as experienced by nurses caring for ASCT patients. This demonstrates that the washing protocol reduced the impact of a number of ASCT related side effects from the point of view of patients, nurses and carers.

As the majority of ASCTs performed at the Royal Hobart Hospital (RHH) are conducted on an outpatient basis, we are reliant upon carers monitoring the patients in the first phases of the ASCT post reinfusion. A previous study of 22 nurses involved with ASCT described the use of odor avoidance and distancing strategies that potentially compromised the nurse-patient relationship following ASCT [Prior et al., 2000]. This "distancing strategy" is therefore probably applicable to carers as well. Anecdotally, nurses at the RHH have reported headaches, migraines as well as nausea in the presence of the unpleasant smell generated by ASCT patients in the hours following reinfusion of HSC. Hence the washing procedure has the potential to improve the working conditions, and thus the delivery of patient care by both nurses and carers, by reducing the unpleasant smells associated with ASCT.

For completeness, the Wilcoxon rank sum and Welch tests were also applied to the remaining responses for patients, carers and nurses, however, there was no significant difference detected for any of this data at the 24 hour time point. Therefore, secondary analysis to generate potential further hypotheses was restricted to these five "main" VASQOL responses (i.e. unusual smells for nurses and carers and nausea, flushing and unusual tastes for the patients) as there was no evidence that the remaining responses were statistically significant.

Multivariate analysis of variance (MANOVA) is useful in experimental situations, where at least some of the independent variables are manipulated [Azcel, 1999]. However, the MANOVA test requires a complete set of responses for every case, any case that has a missing response is deleted from the analysis. Accordingly, as there were many missing responses from the nurses and the carers, only a restricted analysis could be performed on the combined data from the five main VASQOL responses which suggested evidence of a treatment difference. The responses were log transformed in an attempt to meet the Normality assumption of the MANOVA. However the QQ plots of the residuals from the fits suggested that even after log transformation the responses were not particularly Normally distributed. Therefore, although this data supports the findings of a treatment difference as indicated by the the Wilcoxon rank sum and Welch tests, they are unreliable and so additional analysis was required.

When the MANOVA analysis was restricted to just the patient responses of taste, nausea and flushing, since more data sets were included, the findings of the restricted analysis were more reliable. These findings demonstrate strong evidence that the washing protocol had a significant effect on the patient responses at the 24 hour time point, reducing the duration of these symptoms in comparison to those patients receiving *Unwashed* HSCs.

The changes in response over the 24 hour period following ASCT for the five main VASQOL responses were also analysed with a multivariate analysis of variance. If any response was missing, then all observations for that case were excluded as was the case for the MANOVA test. The majority of the responses do not demonstrate any significant difference as a consequence of the washing protocol over time. Nevertheless, there is evidence of a treatment difference for both nurse smells and patient tastes for all time points. This suggests that the washing protocol reduced the impact of unusual tastes and smells for patients and nurses, respectively over the 24 hour time period.

A particular example of where washing of cells prior to ASCT enhances patient safety are patients with Amyloid light chain (AL) disease. The morbidity and mortality of AL patients undergoing ASCT is higher than for patients undergoing ASCT for other haematological malignancies [Worel et al., 2006]. A number of symptoms have been reported in AL patients following ASCT including gastrointestinal complications (including bleeding and perforation) as well as arrhythmias in patients with cardiac involvement [Worel et al., 2006]. Since DMSO can solubilise amyloid deposits *in-vitro*, the reinfusion of DMSO during the ASCT is an additional concern for AL patients with cardiac involvement [Gertz et al., 2004]. Severe respiratory distress also has been reported in a cardiac amyloid patient within seconds of a autologous reinfusion [Benekli et al., 2000]. The exact mechanism in this case is uncertain as the release of histamine by DMSO or from damaged granulocytes has also been reported to cause various degrees of AV nodal conduction block [Styler et al., 1992] [Benekli et al., 2000]. As a result of the increased risk of reinfusion related toxicities, the washing of HSC post-thawing has been recommended in order to reduce the amount of DMSO reinfused into cardiac amyloid patients [Benekli et al., 2000].

Post-thaw washing of HSC should also be considered as an option for extending the viability of HSC in the event of unexpected delays between thawing and reinfusion. It is also worth considering for those patients who report recent or easily triggered nausea and vomiting, have been heavily conditioned with chemotherapy, or have a smaller body mass (<70 kg) [Synder et al., 2004]. There are also numerous advantages for laboratory and nursing staff. Although the procedure is time consuming for the laboratory staff, once the HSCs have been resuspended post washing, the

time taken for reinfusion at the patient bedside is reduced. Thawing HSCs in the laboratory provides the opportunity to filter platelet clumps before transportation to the ward, therefore removing a cause of delay during reinfusion. Reducing the time required for HSC reinfusion on the ward decreases the time spent by both nursing and laboratory staff performing the procedure, whilst improving the efficacy safety and quality of patient care.

3.5 Summary

This clinical trial provided an invaluable opportunity to assess the impact of the washing protocol for HSC prior to reinfusion on ASCT side effects from the perspective of patients, nurses and carers. Patient observations pre and post ASCT (provided by nursing staff) suggest that there is no adverse effect on patient temperature, urinary haemoglobin and blood pressure in those individuals receiving *Washed* HSC in comparison to *Unwashed*. Additionally, the washing protocol does not appear to increase the incidence of microbiological contamination for undamaged cryobags. Furthermore, the data suggests that washing has an significant impact on reducing the incidence of increased blood pressure associated with ASCT.

Statistical analysis of the VASQOL responses demonstrated evidence of post-thaw washing of HSC improving several symptoms associated with reinfusion including flushing, nausea and unpleasant tastes for patients and unusual smells for both carers and nurses. This suggests that the washing protocol is not only beneficial to patients but also to nursing staff and carers, and subsequently may improve their delivery of patient care.

Consequently, there are a number of circumstances in which a post-thawing strategy is beneficial for the patients. This washing protocol reduces the amount of cellular debris and DMSO reinfused as well as diminishing patient discomfort and therefore should be considered in the event of large volume reinfusions, patient allergies or cardiac issues.

Chapter 4

Discussion

Autologous haemopoetic stem cell transplantation involves the intravenous infusion of the patients own HSCs, collected from BM or PB, to replace diseased BM or to replenish terminally damaged BM after the use of myeloablative therapy [Armitage, 1994]. Cryoprotectants such as DMSO are therefore necessary to protect HSC membrane integrity, and hence survival, by preventing the formation of ice crystals during cryopreservation and subsequent storage in liquid nitrogen [Rowley, 1992]. Consequently, when the patient is reinfused they receive DMSO, ruptured granulocytes and red cell debris, as well as HSC.

The NYCBB post-thaw washing procedure provides a method for washing cryopreserved HSC to remove DMSO (and cellular debris) without affecting the cell viability or clonogenic ability [Rubinstein et al., 1995]. The potential to remove DMSO and cellular debris from cryopreserved HSC prior to reinfusion provides an opportunity to reduce numerous re-infusional side effects such as nausea, vomiting, flushing and increased blood pressure [Davis et al., 1990, Stroncek et al., 1991, Rowley et al., 1999b, Hoyt et al., 2000]. This thesis reports on the laboratory validation of a modified NYCBB method as well as a clinical trial to determine if the procedure is of significant benefit to patients.

Cell loss remains a concern with the NYCBB protocol with one study reporting a median loss of 27.2% (range 11.1 to 41.2%) of total nuclear cells in a study of 12 cord bloods [Antonenas et al., 2001]. This thesis has validated a modified version of the NYCBB method in which the rate of centrifugation was increased from 400 g to 1400 g, in order to improve the separation of the supernatant and cellular pellet. The increased centrifuge speed used in this investigation resulted in improved separation of cell pellets from supernatant, however it also increased the risk of cellular damage. As desired, the removal of the supernatant (containing DMSO, red cell debris and ruptured

granulocytes) by plasma expression was achieved without disturbing the cell pellet. The laboratory validation demonstrated that the modified NYCBB method did not result in any significant loss of cells while improving overall cell viability and reducing the incidence of cell clumping post-thawing.

Multi parameter testing was used to assess the impact of the post-thaw washing protocol on HSC. The enumeration of cells expressing CD34 antigens is the *in-vitro* standard for assessment of stem cell collections engraftment potential, as it provides a rapid indication of HSC numbers [Siena et al., 1991, Keeney et al., 1998]. Viable $CD34^+$analysis was used to determine the number of HSC present using 7AAD (a viability dye) [Keeney et al., 1998]. The finding of no significant difference in viable $CD34^+$HSC counts between *Washed* and *Unwashed* cells demonstrates that the increased centrifugal speed does not adversely affect viable HSC numbers. Colony Forming Units Granulocyte Macrophage (CFU-GM) assays were performed to assess the clonogenic ability of the HSCs to mature into macrophages and granulocytes, an indication of the haematopoietic progenitor content of a stem cell collection [Eaves and Lambie, 1995].

On the basis of these results, the modified NYCBB protocol does not adversely affect the number of viable $CD34^+$HSC or their clonogenic potential. Moreover, the washing protocol appeared to halt the decline in overall cell viability observed over time in *Unwashed* cells while dramatically reducing the level of free haemoglobin and, by extrapolation, DMSO from harvests. Therefore the increased centrifugation rate has improved separation of the cellular pellet and supernatant without damage to the HSCs.

As a consequence of the laboratory validation phase of this thesis, the Flow Cytometry laboratory at the RHH adopted a modification of the ISCT guidelines in which a known number of fluorescent microspheres is used to provide an absolute count direct from the flow cytometer [Keeney et al., 1998]. This removed the need for a WCC from an automated haematology analyser (eliminating a source of potential error), while converting the flow cytometer into a single platform $CD34^+$cell-counting device. Furthermore, we expanded the single platform method for $CD34^+$cell counts by incorporating 7AAD (a viability dye), to identify viable $CD34^+$cells [Keeney et al., 1998]. This method is also now routinely used in our clinical lab for viable $CD34^+$cell counts for post cryopreservation sample analysis.

A non-randomised clinical trial was conducted in order to determine if post-thaw washing of stem cell products prior to reinfusion significantly reduces the morbidity associated with this procedure.

The impact of post-thaw washing of HSC on patient recovery has been successfully investigated with regard to patient engraftment [Nagamura-Inoue et al., 2003, Syme et al., 2004]. A modified version

of a VASQOL survey previously developed [Rowley et al., 1999a] to assess impact of post-thaw washing of symptoms associated with ASCT was used in our clinical trial. Symptoms that were monitored over 24 hours following ASCT include nausea, pain, flushing, nervousness, shortness of breath, unusual smells and tastes. These surveys were completed by patients undergoing ASCT at the Royal Hobart Hospital, as well as both their carer and attending nurse.

Exploratory analysis of the VASQOL responses for all the symptoms previously associated with reinfusion (flushing, nausea, shortness of breath, pain, unusual smells and tastes) [Rowley et al., 1999a] suggested that the most commonly reported side effect following ASCT for patients was unusual tastes although nausea and flushing were also concerns. In addition, unusual smells were the most commonly reported ASCT side effect for both the carers and nurses. The average VASQOL scores for the *Washed* and *Unwashed* groups suggested all of those individuals (patients, nurses and carers) involved with *Washed* ASCT experienced a faster rate of recovery from these ASCT related side effects.

Analysis of the VASQOL observations was challenging due to a huge variability the responses. A possible strategy to reduce variability between subjects in future studies could be the use of a fixed set of observers throughout the VASQOL trial (i.e. nurses). Nevertheless, the data obtained from the VASQOL surveys provide an invaluable opportunity for subjective evaluation of the symptoms associated with *Washed* and *Unwashed* ASCT from the perspective of the patients as well as nurses and carers.

Statistical analysis of the VASQOL responses demonstrated evidence of an improvement in the symptoms highlighted by the exploratory analysis including flushing, nausea and unpleasant tastes for patients and unusual smells for both carers and nurses. It also supported the earlier finding that other symptoms, such as shortness of breath and pain, which have been associated the ASCT appeared to only be a minor concern for individuals in this clinical trial. Therefore, the benefits of the washing protocol to patients are two fold. Firstly, from their perspective there is a reduction in the severity of several symptoms associated with ASCT which is an improvement on current ASCT practise. Secondly, as nursing staff and carers also experience a reduction in unpleasant side effects, the ability of these individuals to deliver patient care should also be improved.

The measurement of ASCT patient physical observations pre and post reinfusion by nursing staff as part of the clinical trial provided necessary insight into the impact of washing HSC on patient well-being. There is no significant difference between individuals receiving *Washed* HSC in comparison to *Unwashed* for patient temperature and urinary haemoglobin. In

addition, the data suggests that washing has an significant impact on reducing the incidence of increased blood pressure associated with ASCT, which is an improvement on the current ASCT practise. Furthermore, all of the samples from *Washed* supernatants taken from undamaged cryobags did not demonstrate any evidence of bacterial contamination as a consequence of the washing protocol.

The two main strategies for reducing ASCT related toxicity can be divided into pre-cryopreservation and post-thawing techniques. As a consequence of pre-cryopreservation improvements over time (i.e. refined harvesting, mobilising and processing techniques) the volume of DMSO and cell lysis products reinfused into patients have already been reduced [Davis et al., 1990]. A previous study of the impact of the NYCBB post-thaw washing procedure on ASCT side effects recommended a pre freezing volume reduction and hence the amount of DMSO used as a more efficacious approach to reducing infusion-related toxicity [Rowley et al., 1999a]. Increasing the NCC during cryopreservation to 5×10^8/ml has accordingly been suggested as a means of reducing DMSO requirements [Rowley et al., 1994]. Thus the volumes of HSC reinfused into patients continue to be reduced over time and remains a primary method of reducing ASCT related toxicity.

Post-thaw washing of HSC should automatically be considered as a means of reducing the increased risks associated with ASCT for patients with heart problems such as cardiac amyloid patients [Benekli et al., 2000]. There are also a number of other circumstances in which the post-thaw removal of DMSO and cellular debris would be useful as in the event of large volume reinfusions or patient allergies (e.g. sulphur) by reducing patient exposure to the deleterious effects of DMSO [Rubinstein et al., 1995]. Furthermore, those patients more likely to experience ASCT related toxicities including individuals with easily triggered nausea and vomiting, patients who have been heavily conditioned with chemotherapy, or have a smaller body mass (<70 kg) would also benefit from the procedure [Synder et al., 2004]. Finally, it should also be considered as a option for extending the viability of HSC in the event of unexpected delays between thawing and reinfusion of HSC [Rubinstein et al., 1995].

The post-thaw washing of HSCs presents a number of advantages for the those individuals caring for ASCT patients and their ability to deliver patient care. Most notably, statistical analysis demonstrated that the working conditions for nurses and carers are improved following the removal of DMSO by reducing the unpleasant smell associated with ASCT. A previous study of oncology nurses caring for ASCT patients documented that exposure to ASCT patients correlated with headaches, nausea, fatigue and patient aversion all of which have the potential to affect patient care [Prior et al., 2000]. There-

fore the removal of DMSO and improvement in working conditions assists nurses and carers in their efforts to care and support patients undergoing ASCT. Thawing HSCs in the laboratory also provides the opportunity to filter platelet clumps before transportation to the ward, therefore removing a cause of delay during reinfusion. Additionally, once the HSCs have been resuspended post-washing, the time taken by laboratory staff and nursing staff to perform the actual reinfusion at the patient bedside is reduced in comparison to current ASCT practise.

The major drawback of the post-thaw HSC washing procedure however is the additional time required by laboratory staff to prepare the cells prior to reinfusion. Studies of automated cell washing devices to wash cord bloods post-thawing have demonstrated that the automation of the NYCBB method is safe and suitable [Rodriguez et al., 2004, Perotti et al., 2004]. Therefore the use of a automated cell washer may overcome the additional workload for laboratory staff and improve the ease of the procedure especially when multiple bags are required to be washed while operating in a closed system [Lemarie et al., 2005]. Co-ordination between the nurses and laboratory staff is also essential prior to the commencement of washing in order to reduce delays in reinfusion of HSCs. The adoption of standard operating procedures for the reinfusion of washed HSCs (incorporating a time line for communication between the laboratory and nursing staff prior to the commencement of thawing) would improve the delivery washed HSCs to patients.

Following evaluation of the data collected from the VASQOL surveys, a number of improvements could be considered for future trials. The removal of symptoms such as nervousness from evaluation by the surveys should be contemplated as the data collected was of minimal use and provided no additional information of the ASCT experience. The impact on laboratory staff of the additional work load and responsibility as a result of the post-thaw washing procedure could also assessed using QOL surveys and workload study tools. Finally, given the time required to gather sufficient data for statistical analysis, a multi centre trial using a common protocol could be considered as a means to recruit a greater number of subjects within a shorter time period.

The laboratory validation study demonstrates that this modified NYCBB post-thaw washing protocol reduces the amount of DMSO and cellular debris reinfused into patients without loss of cell numbers or viability. Furthermore, it does not affect the number of $CD34^{+}$HSCs or their clonogenic potential. The findings of the clinical trial indicate that the post-thaw washing improves the quality of life of patients undergoing ASCT and those individuals caring for them by reducing the impact of symptoms associated with HSC reinfusion. Therefore this method should be recommended as a viable option to reduce reinfusion toxicity of cryopreserved HSC.

This thesis investigated a washing protocol for use with cryopreserved bone marrow and peripheral blood stem cell harvests prior to autologous transplantation. The purpose of washing the stem cells is to reduce the toxicity associated with the reinfusion of the cryoprotectant dimethyl sulfoxide (DMSO), and cellular debris, without compromising the engraftment potential. Parameters investigated in the laboratory validation included; cell loss, overall viability, viable $CD34^{+}$stem cell enumeration, clonogenic potential and reduction in free haemoglobin content. There was no significant loss of cell numbers and overall cell viability was improved significantly by DMSO removal, a factor that correlated with the decreased incidence of cell clumping.

Enumeration of viable $CD34^{+}$HSC by single platform flow cytometry demonstrated that washing did not reduce the number of these crucial cells. Similarly, CFU-GM assays demonstrated that washing did not adversely affect HSC clonogenic potential. Washing also removed greater than 90% of free haemoglobin (from lysed red blood cells) and, by extrapolation, DMSO from harvests. The findings of this study demonstrate that a modified blood stem cell washing protocol is a safe laboratory practice with the potential to reduce the morbidity associated with the reinfusion of cryopreserved stem cells.

This clinical trial investigated the effect of the washing protocol on HSC reinfusion toxicities in comparison to current ASCT practise where cells are reinfused without washing immediately after thawing. Nursing observations demonstrated that washing had no impact on patient temperature and actually prevented the rise in systolic blood pressure observed in the *Unwashed* group. Whilst haemoglobinuria is a commonly quoted reinfusion toxicity in the literature, this was not frequently observed in our *Unwashed* cohort, suggesting that modern apheresis techniques have largely ameliorated this side effect through reducing the number of red blood cells collected and subsequently reinfused. Finally, the possibility of microbiological contamination during the washing procedure also appears limited with none observed in this study.

Statistical analysis of the VASQOL responses demonstrated that washing resulted in an improvement in several reinfusion toxicity symptoms such as flushing, nausea and unpleasant tastes for patients and unusual smells for both carers and nurses. This suggests that the washing protocol is not only beneficial to patients, but also to nursing staff and carers and subsequently may improve their delivery of patient care.

In conclusion, the laboratory validation of a modified NYCBB protocol demonstrates that this procedure does not have any adverse effect on cell numbers or viability, $CD34^{+}$HSC cell counts or clonogenic potential. Basic

nursing observations revealed that washing did not have any pyrogenic effect upon the patient and prevented reinfusion associated hypertension. The clinical trial shows evidence that washing has a significant positive effect on the QOL of patients, nurses and carers in comparison to current ASCT practice by reducing the impact of a number of reinfusion related symptoms. Therefore this washing protocol should be considered for as a alternative to the current ASCT method to reduce the side effects associated with ASCT without compromising patient care.

Chapter 5

Prospective Investigations

Whilst this study has demonstrated that washing of thawed HSC products has the capacity to reduce the side effects associated with the reinfusion of cryopreserved stem cells these findings could be expanded by further investigations.

An important consideration prior to the implementation of the washing protocol used in this study is the time required by laboratory staff to perform the procedure. An evaluation of the impact of the washing protocol on the laboratory staff with regard to time taken and work place stress could be performed via QOL and work effort surveys, thus providing a complete picture of the consequences of the washing protocol of all the individuals involved in the ASCT process. Such a study could provide the basis for the capital expenditure for an automated cell washer.

The automation of the washing protocol via the use of automated cell washers could further enhance the post-thaw HSC washing procedure. Previous studies have reported that the manual procedure may take 3 to 4 hours of work by laboratory staff per patient [Syme et al., 2004]. The procedure reported in this thesis takes approximately 30 minutes to thaw a maximum of two cryobags at a time, hence the post-thaw washing of multiple bags could be time intensive for laboratory staff. Hence, the use of a automated post-thaw washing procedure has been investigated by a number of researchers. Using NYCBB methodology, a laboratory comparison study of 10 manual and 10 automated (using the Sepax S-100, Biosafe) post-thaw cord blood washings was conducted. No significant differences were observed in $CD34^{+}$cell numbers and TNC between the two methods. However, overall processing time was halved and the automated system considerably reducing the required for laboratory staff to wash the HSCs prior to reinfusion [Rodriguez et al., 2004]. Therefore, a comparison study of the automated washing procedure to the manual procedure could investigate both

the impact of automation on the time taken to wash HSC as well as scientist workload. Furthermore, a clinical trial could also be conducted using VASQOL surveys to assess the consequences of automated HSC washing on individuals involved with the reinfusion of HSC in orderto establish if the same positive effects as demonstrated in this thesis are observed.

Bibliography

[Aaronson, 1990] Aaronson, N. K. (1990). Methodologic issues in assessing the quality of life in cancer patients. *Cancer*, 6(3):848–50.

[Aird et al., 1992] Aird, W., Labopin, M., Gorin, N. C., and Antin, J. H. (1992). Long-term cryopreservation of human stem cells. *Bone Marrow Transplant*, 9(6):487–490.

[al Fiar et al., 1997] al Fiar, F., Prince, H. M., Imrie, K., Stewart, A. K., Crump, M., and Keating, A. (1997). Bone marrow mononuclear cell count does not predict neutrophil and platelet recovery following autologous bone marrow transplant: value of the colony-forming unit granulocyte-macrophage (cfu-gm) assay. *Cell Transplant*, 6(5):491–495.

[Anderson, 2002] Anderson, D. (2002). Mosby's medical, nursing, allied health dictionary. Sixth Edition.

[Andrykowski et al., 1995] Andrykowski, M. A., Greiner, C. B., Altmaier, E. M., Burish, T. G., Antin, J. H., Gingrich, R., McGarigle, C., and Henslee-Downey, P. J. (1995). Quality of life following bone marrow transplantation: findings from a multicentre study. *Br J Cancer*, 71(6):1322–1329.

[Antonenas et al., 2001] Antonenas, V., Bradstock, K., Hertzberg, M., Bleakley, M., , and Shaw, P. (2001). Effect of washing procedures on unrelated cord blood units for transplantation in children and adults: Experience at westmead hosptial and the childrens hospital at westmead. *Proceedings of the Bone Marrow Transplant Scientist's Association of Australasia Annual Scientific Meeting.*

[Appelbaum et al., 1978] Appelbaum, F. R., Herzig, G. P., Ziegler, J. L., Graw, R. G., Levine, A. S., and Deisseroth, A. B. (1978). Successful engraftment of cryopreserved autologous bone marrow in patients with malignant lymphoma. *Blood*, 52(1):85–95.

[Apperley et al., 2000] Apperley, J., Gluckman, E., and Gratwohl, A. (2000). *Bone and Marrow Transplantation. The EBMT Handbook.* European School of Hematology, France.

[Armitage, 1994] Armitage, J. O. (1994). Bone marrow transplantation. *N Engl J Med*, 330(12):827–838.

[Ashwood-Smith and Friedmann, 1979] Ashwood-Smith, M. J. and Friedmann, G. B. (1979). Lethal and chromosomal effects of freezing, thawing, storage time, and x-irradiation on mammalian cells preserved at -196 degrees in dimethyl sulfoxide. *Cryobiology*, 16(2):132–140.

[Attarian et al., 1996] Attarian, H., Feng, Z., Buckner, C. D., MacLeod, B., and Rowley, S. D. (1996). Long-term cryopreservation of bone marrow for autologous transplantation. *Bone Marrow Transplant*, 17(3):425–430.

[Audet et al., 1998] Audet, J., Zandstra, P. W., Eaves, C. J., and Piret, J. M. (1998). Advances in hematopoietic stem cell culture. *Curr Opin Biotechnol*, 9(2):146–151.

[Azcel, 1999] Azcel, A. (1999). *Complete Business Statistics.* McGraw-Hill, New York, United States of America.

[Barker et al., 2005] Barker, J., Weisdorf, D., Defor, T., and et al (2005). Transplantation of 2 partially hla-matched umbilical cord blood units to enhance engraftment in adults with hematologic malignancy. *Blood*, 105:1343–47.

[Barlogie et al., 1986] Barlogie, B., Hall, R., Zander, A., Dicke, K., and Alexanian, R. (1986). High-dose melphalan with autologous bone marrow transplantation for multiple myeloma. *Blood*, 67(5):1298–1301.

[Barlogie et al., 1997] Barlogie, B., Jagannath, S., Vesole, D. H., Naucke, S., Cheson, B., Mattox, S., Bracy, D., Salmon, S., Jacobson, J., Crowley, J., and Tricot, G. (1997). Superiority of tandem autologous transplantation over standard therapy for previously untreated multiple myeloma. *Blood*, 89(3):789–793.

[Barlogie et al., 2004] Barlogie, B., Shaughnessy, J., Tricot, G., Jacobson, J., Zangari, M., Anaissie, E., Walker, R., and Crowley, J. (2004). Treatment of multiple myeloma. *Blood*, 103(1):20–32.

[Beaujean et al., 1991] Beaujean, F., Hartmann, O., Kuentz, M., Forestier, C. L., Divine, M., and Duedari, N. (1991). A simple, efficient washing

procedure for cryopreserved human hematopoietic stem cells prior to reinfusion. *Bone Marrow Transplant*, 8(4):291–294.

[Benekli et al., 2000] Benekli, M., Anderson, B., Wentling, D., Bernstein, S., Cruczman, M., and McCarthy, P. (2000). Severe respiratory depression after dimethylsuphoxide-containing autologous stem cell infusion in a patient with al amyloidsois. *Bone Marrow Transplantation*, 25:1299–1301.

[Bezwoda, 1999] Bezwoda, W. R. (1999). Randomised, controlled trial of high dose chemotherapy (hd-cnvp) versus standard dose (caf) chemotherapy for high risk, surgically treated, primary breast cancer. *American Society of Clinical Oncology.*

[Blade et al., 2003] Blade, J., Vesole, D. H., and Gertz, M. (2003). High-dose therapy in multiple myeloma. *Blood*, 102(10):3469–3470.

[Boiron et al., 1996] Boiron, J. M., Reiffers, J., and Lowenthal, R. M. (1996). Blood cell transplantation: past, present and future. *Hematol Cell Ther*, 38(5):399–407.

[Bostrom and Burger, 1999] Bostrom, N. and Burger, S. (1999). Tech talk...infusion of cryopreserved grafts. *Telegraft*, 6:6–7.

[Brayton, 1986] Brayton, C. F. (1986). Dimethyl sulfoxide (dmso): a review. *Cornell Vet*, 76(1):61–90.

[Broxmeyer et al., 1989] Broxmeyer, H. E., Douglas, G. W., Hangoc, G., Cooper, S., Bard, J., English, D., Arny, M., Thomas, L., and Boyse, E. A. (1989). Human umbilical cord blood as a potential source of transplantable hematopoietic stem/progenitor cells. *Proc Natl Acad Sci U S A*, 86(10):3828–3832.

[Brunstein and Wagner, 2006] Brunstein, C. and Wagner, J. (2006). Umbilical cord blood transplantation and banking. *Ann. Rev. Med.*, 57:403–17.

[Buckner et al., 1970] Buckner, C. D., Storb, R., Dillingham, L. A., and Thomas, E. D. (1970). Low temperature preservation of monkey marrow in dimethyl sulfoxide. *Cryobiology*, 7(2):136–140.

[Burnett et al., 1998] Burnett, A. K., Goldstone, A. H., Stevens, R. M., Hann, I. M., Rees, J. K., Gray, R. G., and Wheatley, K. (1998). Randomised comparison of addition of autologous bone-marrow transplantation to intensive chemotherapy for acute myeloid leukaemia in first remission: results of mrc aml 10 trial. uk medical research council adult and children's leukaemia working parties. *Lancet*, 351(9104):700–708.

[Cavins et al., 1962] Cavins, J., Kasakura, S., Thomas, E. D., and Ferrebee, J. W. (1962). Recovery of lethally irradiated dogs following infusion of autologous marrow stored at low temperature in dimethyl-sulfoxide. *Blood*, 20:730–734.

[Cella and Webster, 1999] Cella, D. and Webster, K. A. (1999). Quality of life and treatment value in the management of hematologic. *Semin Oncol*, 26(5 Suppl 14):34–42.

[Cella, 1996] Cella, D. F. (1996). Quality of life outcomes: measurement and validation. *Oncology (Huntingt)*, 10(11 Suppl):233–246.

[Cella and Bonomi, 1995] Cella, D. F. and Bonomi, A. E. (1995). Measuring quality of life: 1995 update. *Oncology (Huntingt)*, 9(11 Suppl):47–60.

[Cella and Tulsky, 1990] Cella, D. F. and Tulsky, D. S. (1990). Measuring quality of life today: methodological aspects. *Oncology (Huntingt)*, 4(5):29–38; discussion 69.

[Cella and Tulsky, 1993] Cella, D. F. and Tulsky, D. S. (1993). Quality of life in cancer: definition, purpose, and method of measurement. *Cancer Invest*, 11(3):327–336.

[Champlin and Gale, 1987] Champlin, R. and Gale, R. P. (1987). Bone marrow transplantation for acute leukemia: recent advances and comparison with alternative therapies. *Semin Hematol*, 24(1):55–67.

[Champlin et al., 2000] Champlin, R. E., Schmitz, N., Horowitz, M. M., Chapuis, B., Chopra, R., Cornelissen, J. J., Gale, R. P., Goldman, J. M., r. Loberiza FR, J., Hertenstein, B., Klein, J. P., Montserrat, E., Zhang, M. J., Ringden, O., Tomany, S. C., Rowlings, P. A., Hoef, M. E. V., and Gratwohl, A. (2000). Blood stem cells compared with bone marrow as a source of hematopoietic cells for allogeneic transplantation. ibmtr histocompatibility and stem cell sources working committee and the european group for blood and marrow transplantation (ebmt). *Blood*, 95(12):3702–3709.

[Chang and Ma, 1998] Chang, A. and Ma, D. (1998). Cd34+ assay in haematopoietic stem cell transplantation. *Today's Life Science*, 10:22–27.

[Chen et al., 2001] Chen, C. I., Crump, M., Tsang, R., Stewart, A. K., and Keating, A. (2001). Autotransplants for histologically transformed follicular non-hodgkin's lymphoma. *Br J Haematol*, 113(1):202–208.

[Cheson et al., 1989] Cheson, B. D., Lacerna, L., Leyland-Jones, B., Sarosy, G., and Wittes, R. E. (1989). Autologous bone marrow transplantation. current status and future directions. *Ann Intern Med*, 110(1):51–65.

[Damiani et al., 1997] Damiani, D., Fanin, R., Silvestri, F., Grimaz, S., Infanti, L., Geromin, A., Cerno, M., Michieli, M., Rinaldi, C., Savignano, C., Trani, G., Fiacchini, M., and Baccarani, M. (1997). Randomized trial of autologous filgrastim-primed bone marrow transplantation versus filgrastim-mobilized peripheral blood stem cell transplantation in lymphoma patients. *Blood*, 90(1):36–42.

[Davis et al., 1990] Davis, J. M., Rowley, S. D., Braine, H. G., Piantadosi, S., and Santos, G. W. (1990). Clinical toxicity of cryopreserved bone marrow graft infusion. *Blood*, 75(3):781–786.

[de Revel et al., 1994] de Revel, T., Appelbaum, F. R., Storb, R., Schuening, F., Nash, R., Deeg, J., McNiece, I., Andrews, R., and Graham, T. (1994). Effects of granulocyte colony-stimulating factor and stem cell factor, alone and in combination, on the mobilization of peripheral blood cells that engraft lethally irradiated dogs. *Blood*, 83(12):3795–3799.

[Desikan et al., 2000] Desikan, R., Barlogie, B., Sawyer, J., Ayers, D., Tricot, G., Badros, A., Zangari, M., Munshi, N. C., Anaissie, E., Spoon, D., Siegel, D., Jagannath, S., Vesole, D., Epstein, J., Shaughnessy, J., Fassas, A., Lim, S., Roberson, P., and Crowley, J. (2000). Results of high-dose therapy for 1000 patients with multiple myeloma: durable complete remissions and superior survival in the absence of chromosome 13 abnormalities. *Blood*, 95(12):4008–4010.

[Dhodapkar et al., 1994] Dhodapkar, M., Goldberg, S. L., Tefferi, A., and Gertz, M. A. (1994). Reversible encephalopathy after cryopreserved peripheral blood stem cell infusion. *Am J Hematol*, 45(2):187–188.

[Dickinson and Company, 2003] Dickinson, B. and Company (2003). Bd procount progenitor cell enumeration kit.

[Eaves and Lambie, 1995] Eaves, C. and Lambie, K. (1995). *Atlas of human hematopoietic colonies*. Stem Cell Technologies, Canada.

[Egorin et al., 1998] Egorin, M. J., Rosen, D. M., Sridhara, R., Sensenbrenner, L., and Cottler-Fox, M. (1998). Plasma concentrations and pharmacokinetics of dimethylsulfoxide and its metabolites in patients undergoing peripheral-blood stem-cell transplants. *J Clin Oncol*, 16(2):610–615.

[Ferrel et al., 1992] Ferrel, B., Grant, M., Schmidt, G., Rhiner, M., Whitehead, C., Fonbuena, P., and Forman, S. (1992). The meaning of quality of life for bone marrow transplant survivors. part 1 the impact of bone marrow transplant on quality of life. *Cancer Nursing*, 15:153–160.

[Ferrell et al., 1992] Ferrell, B., Grant, M., Schmidt, G. M., Rhiner, M., Whitehead, C., Fonbuena, P., and Forman, S. J. (1992). The meaning of quality of life for bone marow transplant survivors. part 2. improving quality of life for bone marrow transplant survivors. *Cancer Nurs*, 15(4):247–53.

[Gaston-Johansson et al., 1992] Gaston-Johansson, F., Franco, T., and Zimmerman, L. (1992). Pain and psychological distress in patients undergoing autologous bone marrow transplantation. *Oncol Nurs Forum*, 19:41–8.

[Gertz et al., 2004] Gertz, M. A., Lacy, M. Q., and Dispenzieri, A. (2004). Therapy for immunoglobulin light chain amyloidosis : the new and the old. *Blood Reviews*, 18:17–37.

[Gillespie and Hillyer, 1996] Gillespie, T. W. and Hillyer, C. D. (1996). Peripheral blood progenitor cells for marrow reconstitution: mobilization and collection strategies. *Transfusion*, 36(7):611–624.

[Goldman et al., 1978] Goldman, J. M., Th'ng, K. H., Park, D. S., Spiers, A. S., Lowenthal, R. M., and Ruutu, T. (1978). Collection, cryopreservation and subsequent viability of haemopoietic stem cells intended for treatment of chronic granulocytic leukaemia in blast-cell transformation. *Br J Haematol*, 40(2):185–195.

[Goodman and Hodgson, 1962] Goodman, J. and Hodgson, G. S. (1962). Evidence for stem cells in the peripheral blood of mice. *Blood*, 19:702–714.

[Gratwohl et al., 2004] Gratwohl, A., Baldomero, H., Demirer, T., Rosti, G., Dini, G., Ladenstein, R., and Urbano-Ispizua, A. (2004). Hematopoetic stem cell transplantation for solid tumors in europe. *Ann Oncol*, 15(4):653–660.

[Hartmann et al., 1985] Hartmann, O., Beaujean, F., Bayet, S., Pico, J. L., Tournade, M. F., Lemerle, L., and Parmentier, C. (1985). Hematopoietic recovery following autologous bone marrow transplantation: role of cryopreservation, number of cells infused and nature of high-dose chemotherapy. *Eur J Cancer Clin Oncol*, 21(1):53–60.

[Hass et al., 1994] Hass, R., Mohle, R., Fruhauf, S., Goldschmidt, H., Witt, B., Flentje, M., Wannenmacher, M., and Hunstein, W. (1994). Patient characteristics associated with successful mobilizing and autografting of peripheral blood progenitor cells in malignant lymphoma. *Blood*, 83(12):3787–3794.

[Hoffman and Pettit, 1989] Hoffman, A. V. and Pettit, J. E. (1989). *Essential Haematology*. Blackwell Scientific Publications, Oxford, London.

[Horowitz et al., 1990] Horowitz, M. M., Gale, R. P., Sondel, P. M., Goldman, J. M., Kersey, J., Kolb, H. J., Rimm, A. A., Ringden, O., Rozman, C., Speck, B., and a. l. .. et (1990). Graft-versus-leukemia reactions after bone marrow transplantation. *Blood*, 75(3):555–562.

[Hoyt et al., 2000] Hoyt, R., Szer, J., and Grigg, A. (2000). Neurological events associated with the infusion of cryopreserved bone marrow and/or peripheral blood progenitor cells. *Bone Marrow Transplant*, 25(12):1285–1287.

[Jacobson et al., 2003] Jacobson, J., Barlogie, B., Shaughnessy, J., Drach, J., Tricot, G., Fassas, A., Zangari, M., Giroux, D., Crowley, J., Hough, A., and Sawyer, J. (2003). Mds-type abnormalities within myeloma signature karyotype (mm-mds): only 13% 1-year survival despite tandem transplants. *Br J Haematol*, 122(3):430–440.

[Jacobson et al., 1951] Jacobson, L., Marks, E. K., Robson, M. J., Gaston, E., and Zirkle, R. E. (1951). The effect of spleen protection on mortality following x-irradiation. *Journal of Laboratory and Clinical Medicine*, 34:1538–1543.

[Janeway et al., 1999] Janeway, C., Travers, P., Walport, M., and Capra, J. D. (1999). *Immunology. The immune system in health and disease. Fourth edition.* Elsevier Science Ltd, Garland Publishing, New York, United States of America.

[Johnson, 1981] Johnson, F. L. (1981). Marrow transplantation in the treatment of acute childhood leukemia. historical development and current approaches. *American Journal of Pediatric Hematology/Oncology*, 3(4):389–395.

[Juttner et al., 1985] Juttner, C. A., To, L. B., Haylock, D. N., Branford, A., and Kimber, R. J. (1985). Circulating autologous stem cells collected in very early remission from acute non-lymphoblastic leukaemia produce

prompt but incomplete haemopoietic reconstitution after high dose melphalan or supralethal chemoradiotherapy. *Br J Haematol*, 61(4):739–745.

[Keeney et al., 1998] Keeney, M., Chin-Yee, I., Weir, K., Popma, J., Nayar, R., and Sutherland, D. R. (1998). Single platform flow cytometric absolute cd34+ cell counts based on the ishage guidelines. international society of hematotherapy and graft engineering. *Cytometry*, 34(2):61–70.

[Kessinger et al., 1990] Kessinger, A., Schmit-Pokorny, K., Smith, D., and Armitage, J. (1990). Cryopreservation and infusion of autologous peripheral blood stem cells. *Bone Marrow Transplant*, 5 Suppl 1:25–27.

[Keung et al., 1994] Keung, Y. K., Lau, S., Elkayam, U., Chen, S. C., and Douer, D. (1994). Cardiac arrhythmia after infusion of cryopreserved stem cells. *Bone Marrow Transplant*, 14(3):363–367.

[King et al., 1995] King, C. R., Ferrell, B. R., Grant, M., and Sakurai, C. (1995). Nurses' perceptions of the meaning of quality of life for bone marrow transplant survivors. *Cancer Nurs*, 18(2):118–29.

[Kollman et al., 2001] Kollman, C., Howe, C. W., Anasetti, C., Antin, J. H., Davies, S. M., Filipovich, A. H., Hegland, J., Kamani, N., Kernan, N. A., King, R., Ratanatharathorn, V., Weisdorf, D., and Confer, D. L. (2001). Donor characteristics as risk factors in recipients after transplantation of bone marrow from unrelated donors: the effect of donor age. *Blood*, 98(7):2043–2051.

[Krishnan et al., 2000] Krishnan, A., Bhatia, S., Slovak, M. L., Arber, D. A., Niland, J. C., Nademanee, A., Fung, H., Bhatia, R., Kashyap, A., Molina, A., O'Donnell, M. R., Parker, P. A., Sniecinski, I., Snyder, D. S., Spielberger, R., Stein, A., and Forman, S. J. (2000). Predictors of therapy-related leukemia and myelodysplasia following autologous transplantation for lymphoma: an assessment of risk factors. *Blood*, 95(5):1588–1593.

[Kurtzberg et al., 1996] Kurtzberg, J., Laughlin, M., Graham, M. L., Smith, C., Olson, J. F., Halperin, E. C., Ciocci, G., Carrier, C., Stevens, C. E., and Rubinstein, P. (1996). Placental blood as a source of hematopoietic stem cells for transplantation into unrelated recipients. *N Engl J Med*, 335(3):157–166.

[Lemarie et al., 2005] Lemarie, C., Calmels, B., Malenfant, C., Arneodo, V., Blaise, D., Viret, F., Bouabdallah, R., Ladaque, P., Viens, P., and Chabannon, C. (2005). Clinical experience with the delivery of thawed and

washed autologous blood cells, with an automated closed fluid management device: Cytomate. *Transfusion*, 45:737–742.

[Lemonnier et al., 1986] Lemonnier, M. P., Gorin, N. C., Laporte, J. P., Douay, L., Lopez, M., van den Akker, J., Stachowiak, J., David, R., Pene, F., Kantor, G., and et al (1986). Autologous marrow transplantation for patients with chronic myeloid leukemia in accelerated or blastic phase: report of 14 cases. *Exp Hematol*, 14(7):654–658.

[Lenhoff et al., 2000] Lenhoff, S., Hjorth, M., Holmberg, E., Turesson, I., Westin, J., Nielsen, J. L., Wisloff, F., Brinch, L., Carlson, K., Carlsson, M., Dahl, I. M., Gimsing, P., Hippe, E., Johnsen, H., Lamvik, J., Lofvenberg, E., Nesthus, I., and Rodjer, S. (2000). Impact on survival of high-dose therapy with autologous stem cell support in patients younger than 60 years with newly diagnosed multiple myeloma: a population-based study. nordic myeloma study group. *Blood*, 95(1):7–11.

[Lewis and Trobaugh, 1964] Lewis, J. and Trobaugh, F. E. (1964). The assay of the transplantation potential of fresh and store bone marrow by two in-vivo systems. *Annals New York Academy of Sciences*, 114:667–685.

[Limaye, 1997] Limaye, L. S. (1997). Bone marrow cryopreservation: improved recovery due to bioantioxidant additives in the freezing solution. *Stem Cells*, 15(5):353–358.

[Lindsley et al., 1955] Lindsley, D., Odell, T. T., and Tausche, F. G. (1955). Implantation of functional erythropoietic elements following total-body irradiation. *Proceedings of the Society for Experimental Biology and Medicine*, 90:512–515.

[Lopez-Jimenez et al., 1994] Lopez-Jimenez, J., Cervero, C., Munoz, A., Hernandez-Madrid, A., Pineda, J. F., Larana, J. G., Moro, C., Maldonado, M., Oteyza, J. P., Otheo, E., and et al (1994). Cardiovascular toxicities related to the infusion of cryopreserved grafts: results of a controlled study. *Bone Marrow Transplant*, 13(6):789–793.

[Lorenz et al., 1951] Lorenz, E., Uphoff, D., Reid, T. R., and et al (1951). Modification of irradiation injury in mice and guinea pigs by bone marrow injections. *J Natl Cancer Inst*, 12:197–201.

[Mach-Pascual et al., 1998] Mach-Pascual, S., Legare, R. D., Lu, D., Kroon, M., Neuberg, D., Tantravahi, R., Stone, R. M., Freedman, A. S., Nadler, L. M., Gribben, J. G., and Gilliland, D. G. (1998). Predictive value

of clonality assays in patients with non-hodgkin's lymphoma undergoing autologous bone marrow transplant: a single institution study. *Blood*, 91(12):4496–4503.

[Malinin et al., 1970] Malinin, T., Pegg, D. E., Perry, V. P., and Brodine, C. E. (1970). Long-term storage of boen marrow cells at liquid nitrogen and dry ice temperatures. *Cryobiology*, 7:65–69.

[Mannick et al., 1960] Mannick, J., Lochte, H. L., Donnall, E., and Ferrebee, J. (1960). In vitro and in vivo assessment of the viability of dog marrow after storage. *Blood*, 15:517–524.

[Martin et al., 2001] Martin, A., Fernandez-Jimenez, M. C., Caballero, M. D., Canales, M. A., Perez-Simon, J. A., de Bustos, J. G., Vazquez, L., Hernandez-Navarro, F., and Miguel, J. F. S. (2001). Long-term follow-up in patients treated with mini-beam as salvage therapy for relapsed or refractory hodgkin's disease. *Br J Haematol*, 113(1):161–171.

[McCreddie et al., 1971] McCreddie, K., Hersh, E. M., and Freireich, E. J. (1971). Cells capable of colony formation in the peripheral blood of man. *J Natl Cancer Inst*, 171:293–294.

[McGlave, 1985] McGlave, P. B. (1985). The status of bone marrow transplantation for leukemia. *Hosp Pract (Off Ed)*, 20(11):97–110.

[McMillan et al., 1990] McMillan, A. K., Goldstone, A. H., Linch, D. C., Gribben, J. G., Patterson, K. G., Richards, J. D., Franklin, I., Boughton, B. J., Milligan, D. W., Leyland, M. M., and et al (1990). High-dose chemotherapy and autologous bone marrow transplantation in acute myeloid leukemia. *Blood*, 76(3):480–488.

[Metayer et al., 2003] Metayer, C., Curtis, R. E., Vose, J., Sobocinski, K. A., Horowitz, M. M., Bhatia, S., Fay, J. W., Freytes, C. O., Goldstein, S. C., Herzig, R. H., Keating, A., Miller, C. B., Nevill, T. J., Pecora, A. L., Rizzo, J. D., Williams, S. F., Li, C. Y., Travis, L. B., and Weisdorf, D. J. (2003). Myelodysplastic syndrome and acute myeloid leukemia after autotransplantation for lymphoma: a multicenter case-control study. *Blood*, 101(5):2015–2023.

[Molineux et al., 1990] Molineux, G., Pojda, Z., Hampson, I. N., Lord, B. I., and Dexter, T. M. (1990). Transplantation potential of peripheral blood stem cells induced by granulocyte colony-stimulating factor. *Blood*, 76(10):2153–2158.

[Mugishima et al., 1999] Mugishima, H., Harada, K., Chin, M., Suzuki, T., Takagi, K., Hayakawa, S., Sato, K., Klein, J. P., and Gale, R. P. (1999). Effects of long-term cryopreservation on hematopoietic progenitor cells in umbilical cord blood. *Bone Marrow Transplant*, 23(4):395–396.

[Nagamura-Inoue et al., 2003] Nagamura-Inoue, T., Shioya, M., Sugo, M., Cui, Y., Takahashi, A., Tomita, S., Zheng, Y., Takada, K., Kodo, H., Asano, S., and Takahashi, T. A. (2003). Wash-out of dmso does not improve the speed of engraftment of cord blood transplantation: follow-up of 46 adult patients with units shipped from a single cord blood bank. *Transfusion*, 43(9):1285–1295.

[Nivison-Smith et al., 2005] Nivison-Smith, I., Hawkins, I., and Ma, D. (2005). *Australasian Bone Marrow Transplant Recipient Registry. Annual Data Summary 2003.* Arrow Foundation, Darlinghurst, NSW.

[Nowell et al., 1956] Nowell, P., Cole, L. J., Habermeyer, J. G., and Roan, P. L. (1956). Growth and continued function of rat marrow cells in x-irradiated mice. *Cancer Research*, 16:258–261.

[Okamoto et al., 1993] Okamoto, Y., Takaue, Y., Saito, S., Shimizu, T., Suzue, T., Abe, T., Sato, J., Hirao, A., Watanabe, T., Kawano, Y., and et al (1993). Toxicities associated with cryopreserved and thawed peripheral blood stem cell autografts in children with active cancer. *Transfusion*, 33(7):578–581.

[Osgood et al., 1939] Osgood, E., Riddle, M. C., and Matthews, T. J. (1939). Aplastic anaemia treated with daily transfusions and intravenous marrow: case report. *Ann Intern Med*, 13:357–67.

[Pedersen-Bjergaard et al., 2000] Pedersen-Bjergaard, J., Andersen, M. K., and Christiansen, D. H. (2000). Therapy-related acute myeloid leukemia and myelodysplasia after high-dose chemotherapy and autologous stem cell transplantation. *Blood*, 95(11):3273–3279.

[Perotti et al., 2004] Perotti, C. G., Fante, C. D., Viarengo, G., Papa, P., Rocchi, L., Bergamaschi, P., Bellotti, L., Marchesi, A., and Salvaneschi, L. (2004). A new automated cell washer device for thawed cord blood units. *Transfusion*, 44(6):900–906.

[Perry et al., 1999] Perry, A. R., Peniket, A. J., Watts, M. J., Leverett, D., Goldstone, A. H., and Linch, D. C. (1999). Peripheral blood stem cell versus autologous bone marrow transplantation for hodgkin's disease:

equivalent survival outcome in a single-centre matched-pair analysis. *Br J Haematol*, 105(1):280–287.

[Philip et al., 1995] Philip, T., Guglielmi, C., Hagenbeek, A., Somers, R., der Lelie, H. V., Bron, D., Sonneveld, P., Gisselbrecht, C., Cahn, J. Y., Harousseau, J. L., and et al (1995). Autologous bone marrow transplantation as compared with salvage chemotherapy in relapses of chemotherapy-sensitive non-hodgkin's lymphoma. *N Engl J Med*, 333(23):1540–1545.

[Prior et al., 2000] Prior, D., Mitchell, A., Nebauer, M., and Smith, M. (2000). Oncology nurses' experience of dimethyl sulfoxide odor. *Cancer Nurs*, 23(2):134–140.

[Ragab et al., 1977] Ragab, A. H., Gilkerson, E., and Myers, M. (1977). Factors in the cryopreservation of bone marrow cells from children with acute lymphocytic leukemia. *Cryobiology*, 14(2):125–134.

[Rock et al., 2000] Rock, G., Chin-Yee, I., Cantin, G., Giulivi, A., Gluck, S., Keating, A., Keeny, M., Klassen, J., and Sutherland, R. (2000). Quality assurance of progenitor cell content of apheresis products: a comparison of clonogenic assays and cd34+ enumeration. the canadian apheresis group and canadian bone marrow. *Transfus Med*, 10(1):67–75.

[Rodriguez et al., 2004] Rodriguez, L., Azqueta, C., Azzalin, S., Garcia, J., and Querol, S. (2004). Washing of cord blood grafts after thawing: high cell recovery using an automated and closed system. *Vox Sang*, 87(3):165–172.

[Rowley et al., 1999a] Rowley, S., Feng, Z., Yadock, D., Holmberg, L., Macleod, B., and Heimfeld, S. (1999a). Post-thaw removal of dmso does not completely abrogate infusional toxicity or the need for pre-infusion histamine blockade. *Cytotherapy*, 1:439–446.

[Rowley et al., 1999b] Rowley, S., Macleod, B., Heimfeld, S., Holmberg, L., and Bensinger, W. I. (1999b). Severe central nervous system toxicity asociated with the infusion of cryopreserved pbsc components. *Cytotherapy*, 1:311–317.

[Rowley, 1992] Rowley, S. D. (1992). Hematopoietic stem cell cryopreservation: a review of current techniques. *J Hematother*, 1(3):233–250.

[Rowley and Anderson, 1993] Rowley, S. D. and Anderson, G. L. (1993). Effect of dmso exposure without cryopreservation on hematopoietic progenitor cells. *Bone Marrow Transplant*, 11(5):389–393.

[Rowley et al., 1994] Rowley, S. D., Bensinger, W. I., Gooley, T. A., and Buckner, C. D. (1994). Effect of cell concentration on bone marrow and peripheral blood stem cell cryopreservation. *Blood*, 83(9):2731–2736.

[Rubinstein et al., 1995] Rubinstein, P., Dobrila, L., Rosenfield, R. E., Adamson, J. W., Migliaccio, G., Migliaccio, A. R., Taylor, P. E., and Stevens, C. E. (1995). Processing and cryopreservation of placental/umbilical cord blood for unrelated bone marrow reconstitution. *Proc Natl Acad Sci U S A*, 92(22):10119–10122.

[Ruutu et al., 2000] Ruutu, T., Volin, L., Parkkali, T., Juvonen, E., and Elonen, E. (2000). Cyclosporine, methotrexate, and methylprednisolone compared with cyclosporine and methotrexate for the prevention of graft-versus-host disease in bone marrow transplantation from hla-identical sibling donor: a prospective randomized study. *Blood*, 96(7):2391–2398.

[Rybka et al., 1980] Rybka, W. B., Mittermeyer, K., Singer, J. W., Buckner, C. D., and Thomas, E. D. (1980). Viability of human marrow after long-term cryopreservation. *Cryobiology*, 17(5):424–428.

[Saleh and Brockopp, 2001] Saleh, U. S. and Brockopp, D. Y. (2001). Quality of life one year following bone marrow transplantation: psychometric evaluation of the quality of life in bone marrow transplant survivors tool. *Oncol Nurs Forum*, 28(9):1457–1464.

[Samson and Singer, 2001] Samson, D. and Singer, C. (2001). Multiple myeloma. *Clin Med*, 1(5):365–370.

[Santos, 1983] Santos, G. W. (1983). History of bone marrow transplantation. *Clin Haematol*, 12(3):611–639.

[Schimmer et al., 2000] Schimmer, A. D., Jamal, S., Messner, H., Keating, A., Meharchand, J., Huebsch, L., Walker, I., Benger, A., Gluck, S., and Smith, A. (2000). Allogeneic or autologous bone marrow transplantation (bmt) for non-hodgkin's lymphoma (nhl): results of a provincial strategy. ontario bmt network, canada. *Bone Marrow Transplant*, 26(8):859–864.

[Schmitz et al., 1996] Schmitz, N., Gratwohl, A., and Goldman, J. M. (1996). Allogeneic and autologous transplantation for haematological diseases, solid tumours and immune disorders. current practice in europe in 1996 and proposals for an operational classification. accreditation subcommittee of the european group for blood and marrow transplantation (ebmt). *Bone Marrow Transplant*, 17(4):471–477.

[Siena et al., 1991] Siena, S., Bregni, M., Brando, B., Belli, N., Ravagnani, F., Gandola, L., Stern, A. C., Lansdorp, P. M., Bonadonna, G., and Gianni, A. M. (1991). Flow cytometry for clinical estimation of circulating hematopoietic progenitors for autologous transplantation in cancer patients. *Blood*, 77(2):400–409.

[Spitzer et al., 1980] Spitzer, G., Verma, D. S., Fisher, R., Zander, A., Vellekoop, L., Litam, J., McCredie, K. B., and Dicke, K. A. (1980). The myeloid progenitor cell–its value in predicting hematopoietic recovery after autologous bone marrow transplantation. *Blood*, 55(2):317–323.

[Stevens, 2001] Stevens, J. (2001). *Applied multivariate statistics for the social sciences.* Lawrence Erlbaum. USA.

[Stewart et al., 1989] Stewart, F. M., Kaiser, D. L., Ishitani, K. P., Pirsch, G. W., and Niskanen, E. (1989). Progenitor cell numbers (cfu-gm, cfu-d, and cfu-mix) and hemopoietic recovery following autologous marrow transplantation. *Exp Hematol*, 17(9):974–980.

[Storb et al., 1970] Storb, R., Epstein, R. B., Graham, T. C., and Thomas, E. D. (1970). Methotrexate regimens for control of graft-versus-host disease in dogs with allogeneic marrow grafts. *Transplantation*, 9(3):240–246.

[Stroncek et al., 1991] Stroncek, D. F., Fautsch, S. K., Lasky, L. C., Hurd, D. D., Ramsay, N. K., and McCullough, J. (1991). Adverse reactions in patients transfused with cryopreserved marrow. *Transfusion*, 31(6):521–526.

[Styler et al., 1992] Styler, M. J., Topolsky, D. L., Crilley, P. A., Covalesky, V., Bryan, R., Bulova, S., and Brodsky, I. (1992). Transient high grade heart block following autologous bone marrow infusion. *Bone Marrow Transplant*, 10(5):435–438.

[Sullivan and Storb, 1984] Sullivan, K. M. and Storb, R. (1984). Allogeneic marrow transplantation. *Cancer Invest*, 2(1):27–38.

[Swanson, 1985] Swanson, B. N. (1985). Medical use of dimethyl sulfoxide (dmso). *Rev Clin Basic Pharm*, 5(1-2):1–33.

[Syme et al., 2004] Syme, R., Berwick, M., Stewart, D., Porter, K., Chadderton, T., and Gluck, S. (2004). The role of depletion of dimethyl sulfoxide before autografting: On hematologic recovery, side effects, and toxicity. *Biology of Blood and Marrow Transplantation*, 10:135–141.

[Synder et al., 2004] Synder, E., Haley, N., and D.J.Triulzi (2004). *Cellular Therapy. A Physician's Handbook.* AABB, Bethesda, Maryland, USA.

[Tegg et al., 1999] Tegg, E. M., Tuck, D. M., Lowenthal, R. M., and Marsden, K. A. (1999). The effect of g-csf on the composition of human bone marrow. *Clin Lab Haematol*, 21(4):265–270.

[Thomas and Ferrebee, 1962] Thomas, E. and Ferrebee, J. W. (1962). Prolonged storage of marrow and its use in the treatment of radiation injury. *Transfusion*, 2:115–117.

[Thomas et al., 1957] Thomas, E., Lochte, H. L., Lu, W. C., and Ferrebee, J. W. (1957). Intravenous infusion of bone marrow in patients recieving radiation and chemotherapy. *New Eng. J. Med*, 257:491–496.

[Thomas, 2000] Thomas, E. D. (2000). Landmarks in the development of hematopoietic cell transplantation. *World J Surg*, 24(7):815–818.

[Thomas and Storb, 1970] Thomas, E. D. and Storb, R. (1970). Technique for human marrow grafting. *Blood*, 36(4):507–515.

[Thomas et al., 1975] Thomas, E. D., Storb, R., Clift, R. A., Fefer, A., Johnson, L., Neiman, P. E., Lerner, K. G., Glucksberg, H., and Buckner, C. D. (1975). Bone-marrow transplantation (second of two parts). *N Engl J Med*, 292(17):895–902.

[To et al., 1998] To, B., Shepherd, M. K., Lam-Po-Tang, R., Szer, J., and McGrath, K. M. (1998). Guidelines for the collection, processing, storage and of administration of hemopoietic stem and progenitor cells for transplantation. report of the working party on hemopoietic stem cell processing, hematology discipline advisory committee, the royal college of pathologists of australasia. *Pathology*, 30:276–285.

[To et al., 1984] To, L. B., Haylock, D. N., Kimber, R. J., and Juttner, C. A. (1984). High levels of circulating haemopoietic stem cells in very early remission from acute non-lymphoblastic leukaemia and their collection and cryopreservation. *Br J Haematol*, 58(3):399–410.

[To et al., 1997] To, L. B., Haylock, D. N., Simmons, P. J., and Juttner, C. A. (1997). The biology and clinical uses of blood stem cells. *Blood*, 89(7):2233–2258.

[To et al., 1990] To, L. B., Shepperd, K. M., Haylock, D. N., Dyson, P. G., Charles, P., Thorp, D. L., Dale, B. M., Dart, G. W., Roberts, M. M., Sage,

R. E., and et al (1990). Single high doses of cyclophosphamide enable the collection of high numbers of hemopoietic stem cells from the peripheral blood. *Exp Hematol*, 18(5):442–447.

[Tsimberidou et al., 2003] Tsimberidou, A. M., Stavroyianni, N., Viniou, N., Papaioannou, M., Tiniakou, M., Marinakis, T., Skandali, A., Sakellari, I., and Yataganas, X. (2003). Comparison of allogeneic stem cell transplantation, high-dose cytarabine, and autologous peripheral stem cell transplantation as postremission treatment in patients with de novo acute myelogenous leukemia. *Cancer*, 97(7):1721–1731.

[van der Wall et al., 1994] van der Wall, E., Richel, D. J., Holtkamp, M. J., Slaper-Cortenbach, I. C., van der Schoot, C. E., Dalesio, O., Nooijen, W. J., Schornagel, J. H., and Rodenhuis, S. (1994). Bone marrow reconstitution after high-dose chemotherapy and autologous peripheral blood progenitor cell transplantation: effect of graft size. *Ann Oncol*, 5(9):795–802.

[Vellenga et al., 2001] Vellenga, E., van Agthoven, M., Croockewit, A. J., Verdonck, L. F., Wijermans, P. J., van Oers, M. H., Volkers, C. P., van Imhoff, G. W., Kingma, T., de Groot, C. A. U., and Fibbe, W. E. (2001). Autologous peripheral blood stem cell transplantation in patients with relapsed lymphoma results in accelerated haematopoietic reconstitution, improved quality of life and cost reduction compared with bone marrow transplantation: the hovon 22 study. *Br J Haematol*, 114(2):319–326.

[Verdonck et al., 1997] Verdonck, L. F., Dekker, A. W., Lokhorst, H. M., Petersen, E. J., and Nieuwenhuis, H. K. (1997). Allogeneic versus autologous bone marrow transplantation for refractory and recurrent low-grade non-hodgkin's lymphoma. *Blood*, 90(10):4201–4205.

[Vose et al., 2001] Vose, J. M., Zhang, M. J., Rowlings, P. A., Lazarus, H. M., Bolwell, B. J., Freytes, C. O., Pavlovsky, S., Keating, A., Yanes, B., van Besien, K., Armitage, J. O., and Horowitz, M. M. (2001). Autologous transplantation for diffuse aggressive non-hodgkin's lymphoma in patients never achieving remission: a report from the autologous blood and marrow transplant registry. *J Clin Oncol*, 19(2):406–413.

[Wells et al., 1979] Wells, J. R., Ho, W. G., Graze, P., Sullivan, A., Gale, R. P., and Cline, M. J. (1979). Isolation, cryopreservation, and autotransplantation of human stem cells. *Exp Hematol*, 7 Suppl 5:12–20.

[Whedon et al., 1995] Whedon, M., Stearns, D., and Mills, L. E. (1995). Quality of life of long-term adult survivors of autologous bone marrow transplantation. *Oncology Nursing Forum*, 22(10):1527–1537.

[Willams et al., 1990] Willams, W., Beutler, E., Erslev, A. J., and Lichtman, M. A. (1990). *Hematology*. McGraw-Hill, New York, United States of America.

[Witherspoon et al., 1989] Witherspoon, R. P., Fisher, L. D., Schoch, G., Martin, P., Sullivan, K. M., Sanders, J., Deeg, H. J., Doney, K., Thomas, D., Storb, R., and et al (1989). Secondary cancers after bone marrow transplantation for leukemia or aplastic anemia. *N Engl J Med*, 321(12):784–789.

[Worel et al., 2006] Worel, N., andM. Mitterbauer, A. S., Keil, F., Rabitsch, W., Kalhs, P., Gisslinger, H., Raderer, M., Geissler, K., Hocker, P., Zielinski, C. C., Oberbauer, R., and Greinix, H. T. (2006). Autologous stem-sell transplantation in progressing amyloidosis is associated with severe transplant-related toxicity. *The Middle Europoean Journal of Medicine*, 118/1-2:49–53.

[Zar, 1999] Zar, J. (1999). *Biostatistical analysis*. Prentice Hall. Upper Saddle River, N.J..USA.

[Zittoun et al., 1995] Zittoun, R. A., Mandelli, F., Willemze, R., de Witte, T., Labar, B., Resegotti, L., Leoni, F., Damasio, E., Visani, G., Papa, G., and et al (1995). Autologous or allogeneic bone marrow transplantation compared with intensive chemotherapy in acute myelogenous leukemia. european organization for research and treatment of cancer (eortc) and the gruppo italiano malattie ematologiche maligne dell'adulto (gimema) leukemia cooperative groups. *N Engl J Med*, 332(4):217–223.